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QUANTITATIVE DETERMINATION OF MIXTURES OF ALKYL ETHERS OF D-GLUCOSE

PART II*. STRUCTURAL STUDIES OF PARTIALLY METHYLATED COTTON CELLULOSE

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ABSTRACT

The distribution of methoxyl groups in repeatedly methylated cotton cellulose is in agreement with the suggestion that the elementary fibril is the basic structural element and suggests that a simple model for these elementary fibrils would have an average cross-section of 8×10 cellulose chains. The dimensions of such a cross-section are approximately 40×50 Å and agree closely with electron-microscopical observations. This fibril has a surface in which all of the readily accessible hydroxyl groups lie. Methylation of these hydroxyl groups is quite rapid, but the methylation of hydroxyl groups below this surface takes place at a lower rate. The slower reaction proceeds with little change in methoxyl group distribution, other than the appearance of tri-substituted D-glucose units at a rate similar to that of the disappearance of D-glucose; it is suggested that this stage of the reaction occurs with an advancing zone of methylation passing through ordered material. Evidence has also been obtained for aggregation of the elementary fibrils.

INTRODUCTION

Cellulose has a basically simple molecular structure consisting of D-glucose residues linked β-(1→4) in extremely long chains. The arrangement of these chains in native celluloses is complex. In cotton fibres (diameter ~10–20 μm), a fibrillar structure (diameter ~0.2–0.3 μm) can be seen by optical microscopy. Electron microscopy, however, reveals still smaller structural elements (diameter ~30–60 Å) which are termed elementary fibrils. Cellulose chains (diameter ~5 Å) make up the elementary fibrils, but their arrangement has not yet been fully determined. In these cellulose chains, only a fraction of the hydroxyl groups are accessible to most reagents unless the cellulose is in solution or is in a very highly swollen state. Studies of chemical reactions of cellulose, such as acid hydrolysis and periodate oxidation, suggest that there are areas of differing accessibility. These have been explained in terms of areas of high order (crystalline regions) and areas of disorder (amorphous regions). It has, however, become increasingly difficult to explain all

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TABLE I

DISTRIBUTION OF METHOXYL GROUPS IN REPEATEDLY METHYLATED COTTON CELLULOSE

No. of treatments	*Methoxyl D.S.*	*Moles % of*								*Hydroxyl groups (%) methylated*		
		G[a]	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	*at C-2*	*at C-3*	*at C-6*
1	0.20	86	9	0	3	1	2	0	0	12	2	5
2	0.38	71	9	1	7	2	4	1	3	18	6	13
3	0.56	66	10	1	8	3	6	1	6	25	11	21
5	0.71	58	9	1	9	3	8	1	10	31	15	28
8	0.87	51	9	1	9	3	10	2	14	36	20	35
10	0.92	54	8	1	9	2	9	1	16	35	20	34
15	1.02	53	8	1	9	2	9	1	18	37	22	36
20	1.08	46	7	1	8	2	11	1	22	42	26	41
30	1.29	43	6	1	8	2	10	1	27	45	31	45

[a]G = D-glucose, G_2 = 2-*O*-methyl-D-glucose, etc.

observations in these terms, and the concept of a fibrillar structure, in which the fibrils may be regarded as crystalline, has gained favour. The structure of cotton cellulose has recently been comprehensively reviewed[1].

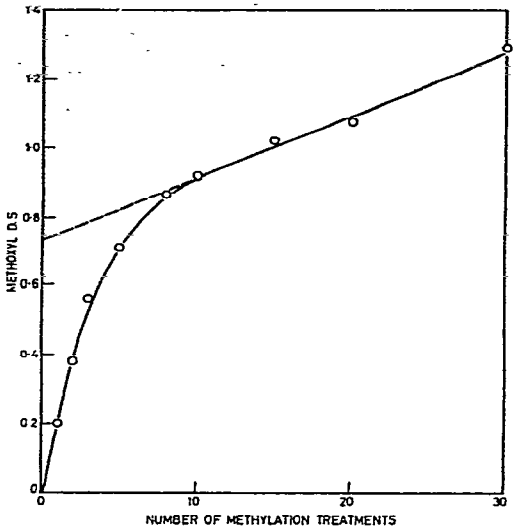
The distribution of methoxyl groups in partially methylated cellulose has been examined previously[2] but only from the point of view of determining the relative reactivities of the three hydroxyl groups present. In this paper, we describe the use of the measured distributions to obtain structural information on cotton cellulose.

RESULTS AND DISCUSSION

Cotton cellulose has been methylated in a repeated treatment which involved soaking in 2N sodium hydroxide, methylation in methyl sulphate–methyl sulphoxide mixtures, washing, and drying. This series of treatments does not lead to extensive swelling of the cellulose as is found with alkali solutions of higher concentration. The extent of reaction was followed by methoxyl determination (Zeisel), either titrimetrically[3] or by a g.l.c. modification[4]. The variation in degree of substitution in relation to the number of methylation treatments may be interpreted as arising from a reaction occurring in two stages (Fig. 1). An initial rapid reaction, which is largely completed in the first five treatments, is followed by a slower reaction which proceeds steadily over successive treatments. Both infrared–deuteration studies (Fig. 2) and moisture-regain measurements (Fig. 3) indicate that the slower reaction is accompanied by little or no change in accessibility. Thus, the cellulose structure is not being disrupted by any swelling due to methylation during this step. During the initial rapid stage of the methylation, there is an increase in accessibility (13% by infrared deuteration and 8% by moisture-regain measurements). This initial swelling action is not a uniform swelling of the cellulose structure but is more likely to be the effect of complete water-swelling together with substitution of accessible hydroxyl groups, leading to a complete separation of the surfaces of the ordered structural elements in the cellulose. The identity of the hydroxyl groups in these surfaces was examined by the hydrolysis of the partly methylated cellulose samples taken during the course of the series of repeated methylation treatments and analysis of the resulting mixture of *O*-methyl-D-glucoses (Table I). The procedure used was that described in Part I[5].

The analysis shows that the main products at all stages of the reaction are D-glucose and 2-, 6-, 2,6-di-, and 2,3,6-tri-*O*-methyl-D-glucoses. The total proportion of the mono- and di-substituted components is approximately constant throughout the later stages of the reaction, suggesting that an advancing zone of methylation is passing through an ordered surface. Initially, the C-2 hydroxyl group is the most reactive (Fig. 4), but, as the reaction proceeds, the extents of methylation at the C-2 and C-6 hydroxyl groups become the same, indicating that equal numbers of these groups are accessible, at least from the eighth methylation onwards. This again suggests that it is an ordered structure which is being methylated. The low reactivity of the C-3 hydroxyl group during the early stages of reaction is noteworthy, and it is

clearly the least accessible hydroxyl group in the later stages of the reaction. This reduced accessibility can be attributed to the involvement of the C-3 hydroxyl group in an intramolecular hydrogen bond[6] to O-5′. It is clear from the observed distri-



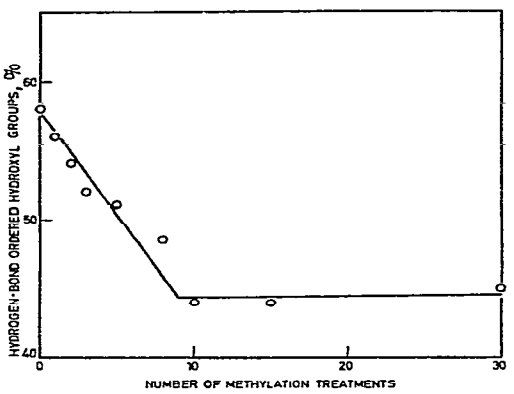
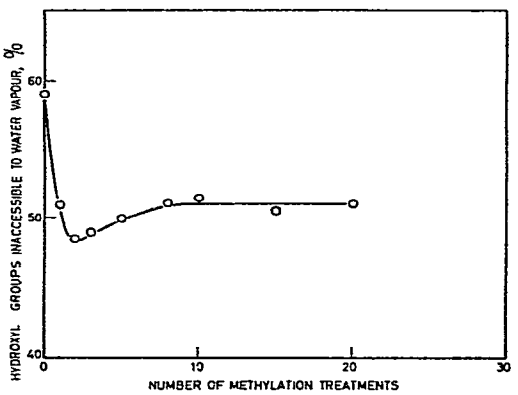


Fig. 1. Repeated methylation of cotton cellulose.

Fig. 2. Changes in degree of hydrogen-bond order in cotton cellulose on repeated methylation, as measured by an infrared deuteration technique.



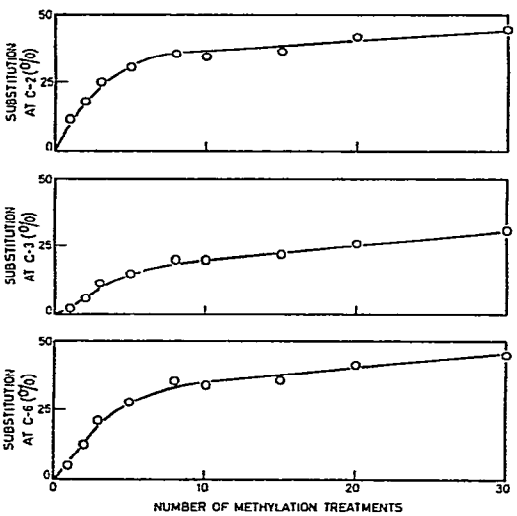


Fig. 3. Changes in accessibility of hydroxyl groups to water vapour in cotton cellulose on repeated methylation.

Fig. 4. Changes in extent of reaction at individual hydroxyl groups during the repeated methylation of cotton cellulose.

butions that reaction at the C-3 hydroxyl group only occurs after the C-2 and C-6 hydroxyl groups have been methylated. This is probably associated with the reduction in intermolecular hydrogen bonding when the C-2 and C-6 hydroxyl groups are substituted; the steric hindrance to methylation of the C-3 hydroxyl group is thereby reduced.

When the proportions of each component are examined in relation to the number of methylation treatments (as in Fig. 5), two stages of change in concentration can be detected, corresponding with the two stages in change of D.S. (degree of substitution). Extrapolation for each component gives the distribution of methoxyl groups at the end of the initial rapid reaction (Table II). Thus, 44% of the D-glucose residues have one or more hydroxyl groups accessible, but only 25% of all of the hydroxyl groups are methylated. The main methylated components, *viz.*, 2-, 6-, 2,6-di- and 2,3,6-tri-*O*-methyl-D-glucose, are present in almost equal amounts.

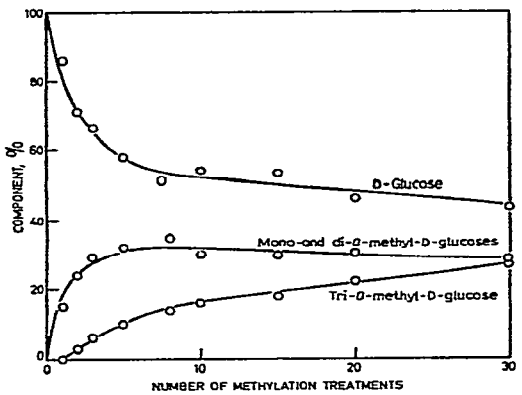


Fig. 5. The extent of mono-, di-, and tri-substitution of cotton cellulose during repeated methylation.

TABLE II

DISTRIBUTION OF METHOXYL GROUPS AT THE END OF THE INITIAL RAPID REACTION IN REPEATED METHYLATION OF CELLULOSE

	Moles % of								
	G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	*D.S.*
Cotton cellulose	56	10	1	10	2	10	1	9	0.76
2N NaOH–Me_2SO_4–Me_2SO									
Expected distribution on basis of model	60	10	—	10	—	10	—	10	0.70

If the D-glucose arises from residues which are inaccessible by virtue of lying beneath a single layer of accessible residues, and the cellulose chains containing the residues are in a rectangular bundle, then it is possible to calculate the size of models of cross-sections one D-glucose residue thick which would give rise to the observed numbers of inaccessible residues (Table III). However, the cross-sectional model cannot be defined uniquely in this way.

TABLE III

INACCESSIBLE D-GLUCOSE RESIDUES IN SIMPLE MODEL CROSS-SECTIONS OF VARYING SIZE[a]

		Inaccessible D-glucose residues (%)						
No. of D-glucose residues in Side A	12	55	60	62	65	67	68	69
	11	54	58	61	63	66	67	68
	10	52	57	60	62	64	66	67
	9	52	56	58	60	62	63	65
	8	50	54	56	58	60	61	62
	7	48	51	54	56	57	58	60
	6	44	48	50	52	53	54	55
		6	7	8	9	10	11	12
		No. of D-glucose residues in side B						

[a]Rectangular cross-sections are assumed. In this calculation, a D-glucose residue is assumed inaccessible if not in the surface layer.

Considering the individual D-glucose residues in a cellulose chain which forms part of a surface, the orientation of the D-glucose residues within that surface will dictate the accessibility of the hydroxyl groups. It is to be expected that D-glucose residues lying mainly in the plane of the surface will have a different distribution of accessible hydroxyl groups from those at right angles to the surface. Thus, it can be assumed that the products of methylation of the two pairs of sides of the cross-sectional model will be different. If it is assumed that all four major products are not formed in any one side, then the proportions of these components found ($\sim$10% of each) suggest that the cross-section will be either almost square or have sides in the approximate ratio of 1:3. From a calculation of the numbers of accessible D-glucose residues in the sides of almost square cross-sections (Table IV), it can be seen that equal numbers of D-glucose residues in sides A and B are only given by cross-sections of 11×12, 10×12, 9×11, 8×10, 7×9, and 6×8. Of these models, all but those of 8×10 and 7×9 can be discounted as they require quantities of inaccessible D-glucose residues considerably removed from the 56% found experimentally. In the case where the sides are in the approximate ratio of 1:3, a similar calculation can be made, and cross-sections of 6×4, 4×14, 9×5, 5×17, 12×6, 6×20, 15×7, and 7×23 would fit this requirement. It is possible to eliminate all but three of these cross-sections (5×17, 12×6, and 6×20) on the basis of their requirement of inaccessible D-glucose residues differing from the 56% found experimentally.

The remaining models (5×17, 12×6, 6×20, 7×9, and 8×10) all have side lengths between 25 Å and 100 Å, which are within the range of dimensions of the elementary fibril as measured by electron microscopy[7]. Thus, the observed extent of reaction is consistent with the methylation of the elementary fibrillar surfaces. Although the reported observations[7] of the elementary fibrillar dimensions tend to

favour approximately square cross-sections, it is not possible to differentiate between the five most likely models.

TABLE IV

ACCESSIBLE D-GLUCOSE RESIDUES IN THE SIDES OF SIMPLE MODEL CROSS-SECTION OF VARYING SIZE[a]

No. of D-glucose residues in Side A	*% of D-glucose residues in Side A; in Side B*						
12	17;6	14;6	13;6	11;7	10;7	9;7	8;7
11	17;6	14;7	13;7	11;7	10;7	9;7	8;8
10	17;7	14;7	13;8	11;8	10;8	9;8	8;8
9	17;7	14;8	13;8	11;9	10;9	8;8	8;9
8	17;8	14;9	13;9	11;10	10;10	9;10	8;10
7	17;10	14;10	13;11	11;11	10;11	9;12	8;12
6	17;11	14;12	13;13	11;13	10;13	9;14	8;14
	6	7	8	9	10	11	12

No. of D-glucose residues in Side B

[a]Rectangular cross-sections are assumed. Cellulose chains with D-glucose residues common to both sides, *i.e.*, corner units are assumed for the calculation to lie in Side A.

To define the model more closely, we must further consider the orientation of the cellulose chains. A single cellulose chain has an extended, ribbon-like conformation in which the D-glucose residues are bonded not only by the β-D-(1→4)-glucosidic bond but also by the C-3(OH)–O-5′ hydrogen bond[8]. If the chain lies in the surface of a structural element such that the plane of the surface is at right angles to the plane of the D-glucose residues and along the chain axis (*i.e.*, in the 101 crystallographic plane), then on alternate D-glucose residues the C-6 hydroxyl and the C-2 and C-3 hydroxyl groups would be expected to be accessible. As little 2,3-di-*O*-methyl-D-glucose has been found, D-glucose residues having both C-2 and C-3 hydroxyl groups accessible cannot be present. If, however, the involvement of the C-3 hydroxyl group in a hydrogen bond with O-5′ is such that it is rendered inaccessible, then the alternate D-glucose residues would be expected to have either C-2 or C-6 hydroxyl groups accessible. In the remaining pair of sides, surfaces containing the anhydroglucose rings (*i.e.*, the 10$\bar{1}$ crystallographic planes) might be expected, from consideration of molecular models, to contain D-glucose residues having all three hydroxyl groups accessible to methylation. Closer examination of the models reveals that the C-3 hydroxyl group, if involved in a hydrogen bond with the adjacent O-5′, would be orientated above and below the surface plane on alternate pairs of D-glucose residues along the cellulose chain. When orientated below the surface, it is in a position which is considerably crowded, particularly when the adjacent C-2 and C-6′ hydroxyl groups are methylated (Fig. 6). It is not possible to obtain a complete picture of the molecular alignments in this area due to lack of precise knowledge of the arrangement of parallel cellulose chains, but it is clear that the

C-3 hydroxyl group is not readily accessible from the surface and that its methylation would involve some disruption of the structure. Thus, the C-3 hydroxyl group on alternate D-glucose residues along the chain would be expected to be inaccessible to methylation in the initial, rapid reaction, so that equal numbers of D-glucose residues having either C-2 and C-6, or C-2, C-3, and C-6 hydroxyl groups accessible would be found in this pair of surfaces. Thus, four main components are to be expected, equal quantities of 2-, or 6-*O*-methyl-D-glucose from one pair of surfaces, and equal quantities of 2,6-di- and 2,3,6-tri-*O*-methyl-D-glucose from the other pair. It is clear therefore that the cross-sectional models based on the 3:1 ratio of side lengths are not compatible with the expected distribution of methoxyl groups; this leaves the 7×9 and 8×10 cross-sections.

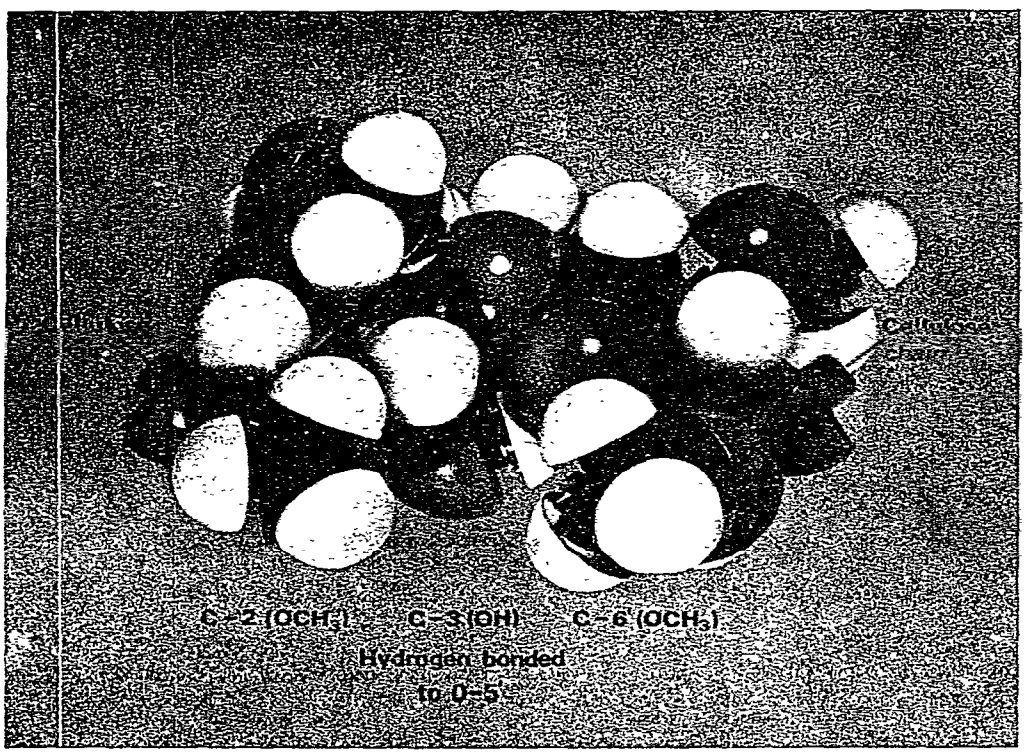


Fig. 6. Molecular model of a cellobiose unit in a cellulose chain, showing the relative inaccessibility of the buried C-3 hydroxyl group to methylation.

On balance, the 8×10 D-glucose residue cross-section forms the most satisfactory approximation. This model requires 60% of the D-glucose residues to be inaccessible in the initial, rapid stage of the methylation which is rather higher than the observed value (56%); any imperfections in the structure would lead to additional methylated products, thus decreasing the total content of D-glucose. More importance is attached to the agreement between the observed total proportion of methylated

components (39%) and that required by the model (40%). The size of an 8×10 cross-section would be about 40×50 Å, closely approximating to the lower limit of size of the elementary fibril observed by electron microscopy[7]. (It should be noted that the cross-section of size 8×10 is now preferred to that of 8×12 previously quoted[9-11]. The larger size was, in part, based on the consideration of possibilities of aggregation of such units; this is now seen to be erroneous, and the 8×10 cross-section represents the best fit in terms of the ratio of accessible to inaccessible D-glucose residues and of the distribution of accessible hydroxyl groups in the surfaces).

The distribution of methoxyl groups to be expected from this model is as follows. Eight D-glucose residues (10% of the total) give rise to 2,6-di-*O*-methyl-D-glucose and 8 give 2,3,6-tri-*O*-methyl-D-glucose from the two 8 unit sides, and as the residues common to both pairs of sides are considered to be in the sides containing the most accessible residues, 8 residues (10%) give 2-*O*-methyl-D-glucose and 8 give 6-*O*-methyl-D-glucose from the 10 unit sides. This distribution is in excellent agreement with that found experimentally (Table II).

The observed distribution of methoxyl groups can thus be explained in terms of a simple model which is also consistent with the size of the smallest structural elements observed by electron microscopy. This leads to the view that cotton cellulose consists of highly ordered structural elements (elementary fibrils) containing about eighty chains with those D-glucose residues on the surfaces having accessible hydroxyl groups. Reactions occurring under mild swelling conditions (*i.e.*, when cellulose is water swollen) proceed most rapidly at the accessible, surface residues in the elementary fibrils. Further reaction at a lower rate occurs below these surfaces.

Evidence of aggregation of elementary fibrils has also been found; presumably, common surfaces are held together by hydrogen bonds which are not broken by weak swelling media. This is shown by repeated methylation of cotton cellulose with diazomethane in ether saturated with water. Reaction in dry ether is extremely slow[12], presumably due to almost complete aggregation of the elementary fibrils in the absence of water. In ether saturated with water, the reaction proceeds rapidly over the first five days (by which time, the D.S. is about 0.4) but thereafter much more slowly. The methoxyl distributions in these partially methylated samples (Table V) indicate that the amounts of the most highly methylated D-glucose residues are now much diminished. Plotting the amounts of the individual components and extrapolating the second portion of each curve gives, as before, the amount of each component at the end of the initial rapid stage (Table V).

The rapid stage of the methylation by diazomethane is thus seen to be confined to 13% of all the hydroxyl groups which are situated on 27% of the D-glucose residues. Comparing this with the methyl sulphate methylation, where 26% of all of the hydroxyl groups are methylated (these being situated on 44% of the D-glucose residues), the number of accessible hydroxyl groups is halved, with the most-exposed D-glucose residues (*i.e.*, those giving rise to 2,6-di- and 2,3,6-tri-*O*-methyl-D-glucose in the methyl sulphate methylation) now being less accessible. These results suggest that aggregation is more likely to occur between surfaces which have the most

TABLE V

DISTRIBUTION OF METHOXYL GROUPS DURING DIAZOMETHANE METHYLATION OF COTTON CELLULOSE

Reaction period (days)	*Methoxyl D.S.*	*Moles % of*								*Hydroxyl groups (%) methylated*		
		G	G_2	G_3	G_6	G_{23}	G_{26}	G_{36}	G_{236}	*at C-2*	*at C-3*	*at C-6*
1	0.26	80	6	—	6	2	3	1	1	12	4	11
2	0.35	76	6	—	7	2	4	1	2	14	6	14
3	0.40	73	6	—	7	2	5	1	3	16	7	16
5	0.45	72	7	—	8	2	5	2	4	18	8	19
7	0.42	72	7	—	9	2	5	2	3	16	7	19
10	0.43	72	7	—	8	2	5	2	3	17	8	18
15	0.60	67	7	—	9	2	6	2	7	23	12	25
20	0.54	67	7	—	9	2	5	2	7	21	11	22
Extrapolated to zero time	0.39	73	7	—	9	2	4	1	3	16	6	17

exposed D-glucose residues and which are capable of producing the highest density of hydrogen bonding.

CONCLUSIONS

The change in distribution of methoxyl groups during the repeated methylation of cotton cellulose (wet with 2N sodium hydroxide and methylated with methyl sulphate in methyl sulphoxide) has suggested that, under these conditions, a structural element of cellulose, namely the elementary fibril, is rapidly methylated on its surfaces. A slower reaction, consistent with a zone of methylation passing through ordered material below the surface, also occurs. The observed distributions can be explained in terms of the simple model of a rectangular bundle of cellulose chains with an average cross-section of 8×10 D-glucose residues. The evidence presented here does not permit description of the detailed way in which the chains in the elementary fibrils are arranged, but it is suggested that they are in a regular array. The distribution of the methoxyl groups found in the initial, rapid reaction is compatible with the surfaces expected in such a model. The relative numbers of D-glucose residues having accessible and inaccessible hydroxyl groups are consistent with reaction at the surface of the proposed model. The size of the cross-section 8×10 D-glucose residues, *i.e.*, 40×50 Å, is in good agreement with the dimensions of the elementary fibrils observed by electron microscopy[7].

Reduced accessibility has been found when methylation is by diazomethane in water-saturated ether solution; it is suggested that one third of the surfaces of the elementary fibrils are rendered inaccessible by aggregation. The observed distribution of methoxyl groups suggests that this aggregation occurs most readily through surfaces containing the largest number of accessible hydroxyl groups.

EXPERIMENTAL

Methylation of cotton cellulose with methyl sulphate. — Cotton cellulose in fabric form ($6' \times 6''$, ~35 g) was pretreated by soaking in 2N sodium hydroxide for 45 min and then immersed in a mixture of methyl sulphate (25 ml) and methyl sulphoxide (50 ml) for a further 45 min. [These two operations were conveniently carried out on a laboratory-scale jig (Horsefall Engineering Ltd., Rochdale, Lancashire).] The samples were then rinsed in warm (60°) 1% acetic acid, washed in warm (60°) water for 1 h, and immediately dried at 90° without any intermediate cooling. The treatment was repeated up to 30 times, samples being taken at intermediate steps.

Methylation of cotton cellulose with diazomethane. — Diazomethane was prepared[13] in ether solution from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide. The strength of the solution prepared was *ca.* 0.25M. Cotton cellulose in fabric form ($2'' \times 1\frac{1}{2}''$, ~300 mg) was suspended in diazomethane dissolved in ether saturated with water and kept for 24 h at 0°. The diazomethane solution was replaced daily. Fabric samples were taken at intervals during treatment for 1 to 20 days.

Cotton cellulose. — The sample of cotton cellulose was in fabric form. It had been scoured and bleached to remove non-cellulosic impurities.

Distribution of methoxyl groups. — Analyses were carried out in the manner described in the previous paper[5].

Infrared deuteration and moisture regain. — Accessibility measurements using these techniques were made in the manner described previously[10].

ACKNOWLEDGMENTS

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SOME STRUCTURAL FEATURES OF *Panax ginseng* C. A. MEY PECTIN

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ABSTRACT

The pectin isolated from roots of *Panax ginseng* C. A. Mey, contains residues of D-galacturonic acid, D-galactose, and L-arabinose, as main components, and those of D-xylose, L-rhamnose, and an unidentified sugar, as minor components of its carbohydrate chain. On partial hydrolysis with acid, the pectin afforded a galacturonan having a linear chain of (1→4)- and, possibly, (1→5)-linked α-D-galacturonic acid residues. Pectinase digestion of the pectin furnished a neutral, pectic polysaccharide in which L-arabinofuranose residues apparently occur in the exterior chains. On partial hydrolysis with acid, the polysaccharide afforded a branched galactan and an acidic heteropolysaccharide. The galactan contains chains of (1→3)- and (1→6)-linked D-galactopyranose residues. The heteropolysaccharide appears to contain sequences involving D-galactose residues, and others involving residues of D-galacturonic acid, D-xylose, and L-rhamnose.

INTRODUCTION

Recently, we reported[1] the isolation of a polysaccharide mixture, containing a starch-like glucan and a pectin, from the roots of *Panax ginseng* C. A. Mey. A preliminary investigation of the pectin showed that it contained D-galacturonic acid, D-galactose, and L-arabinose as main components, and D-xylose, L-rhamnose, and an unidentified sugar as minor components.

The present paper describes the elucidation of some structural features of *Panax ginseng* pectin.

EXPERIMENTAL

General experimental conditions. — Partition chromatography was performed on Whatman 3 MM or Leningrad factory "Goznak" paper with the following solvent systems (v/v): (*A*) ethyl acetate–pyridine–water–acetic acid (5:5:3:1); (*B*) butanone saturated with 1% ammonia; and (*C*) ethyl acetate–acetic acid–water (18:7:8). Thin-layer chromatography (t.l.c.) of methylated sugar derivatives was performed on silica gel "KSK" (> 200 mesh) with chloroform–methanol (10:1) as developer.

T.l.c. of sugars was performed as described elsewhere[2]. Detection was effected with aniline hydrogen phthalate, silver nitrate in ammonia at 105°/10 min, and conc. sulphuric acid or saturated aqueous ammonium sulphate at 115°/10 min. Gas–liquid chromatography (g.l.c.) was carried out as previously described[3]. Pretreated[4] DEAE-cellulose (phosphate form) was used for ion-exchange chromatography. Molecular-sieve chromatography (m.s.c.) was carried out on Biogel P columns[5]. The columns were calibrated with dextrans of known molecular weights. I.r.-spectra were recorded on a Zeiss UR-10 spectrometer. Uronic acid contents were determined by a modified carbazole reaction[6], and protein by the Lowry method[7]. Ash content was determined by heating polysaccharide samples to constant weight at 600°. The methoxyl content was determined by the Vieböck and Schwappach method[8]. The samples were pre-treated with water vapour to remove methanol[9]. The periodate consumption was determined spectrophotometrically[10]. All solutions were concentrated *in vacuo* at 30–40° in a rotatory evaporator.

Chromatography on DEAE-cellulose. — Pectin (300 mg) {$[\alpha]_D^{20}$ +205° (*in* water); OMe, 5.4; uronic acid, 60.0; protein, 3.8; ash, 8.9%} in water (15 ml) was added to a column (3.6 × 40 cm) containing DEAE-cellulose in the phosphate form. The column was eluted in succession with 200 ml each of water, 0.01M, 0.025M, 0.05M, 0.10M, 0.25M, and 0.5M sodium dihydrogen phosphate, and, finally, with a gradient of water–0.3M sodium hydroxide (1 litre). The fractions (15 ml) were analysed by the phenol–sulphuric acid method[11]. After dialysis, polysaccharide material was recovered in the usual way. Three fractions were obtained: 0.01M sodium dihydrogen phosphate eluted a negligible amount of polysaccharide; 0.25M sodium dihydrogen phosphate eluted polysaccharide (0.06 g) containing galacturonic acid, galactose, arabinose, xylose, and rhamnose residues; and sodium hydroxide eluted polysaccharide (0.10 g) having the same qualitative composition of monosaccharides.

Periodate oxidation of the pectin. — The pectin (1.26 g) was added to sodium metaperiodate which was prepared from periodic acid (2.88 g) and cold acetate buffer (pH *ca.* 3.8). The reaction mixture was kept in the dark at 4° with periodic shaking over 12 days. The consumption of periodate was monitored in the usual way on aliquots. When the reaction was complete, ethylene glycol was added to destroy the residual periodate. After deionization, the solution was treated with potassium borohydride (1 g) for 24 h, deionized, and evaporated. The residue (0.71 g) was hydrolysed with 2N sulphuric acid for 10 h at 100°. The hydrolysate was examined by paper chromatography (solvent *A*) and t.l.c.

Preparation of a galacturonan. — The pectin (8 g) in water (800 ml) was treated with 2N sulphuric acid (80 ml) for 3 h at 100°. The hydrolysate was then poured into methanol to afford the galacturonan (2.46 g), $[\alpha]_D^{20}$ +240° (water) (Found: OMe, 5.9; uronic acid, 99.0; protein, 0; ash, 1.5%).

Esterification and reduction of the galacturonan. — The galacturonan (1.9 g) was triturated, wetted with methanol, and treated with ethereal diazomethane overnight at 20°. The residue was filtered off and washed with ether. This product (1.88 g) was dissolved in water (75 ml) and reduced with potassium borohydride (1 g)

in water (25 ml). The reaction mixture was kept overnight in a refrigerator, neutralized with acetic acid, dialysed, treated with Amberlite IR-120 (H^+) resin, and evaporated to dryness. Methanol was twice distilled from the residue, and the esterification and reduction procedures were repeated five times to give galactan **1** (0.98 g) (Found: OMe, 17.9; uronic acid, 6.0; protein, 0; ash, 0.66%).

Periodate oxidation, reduction, and hydrolysis of galactan **1**. — The polysaccharide (0.3 g) was oxidised in the dark with 0.05M sodium metaperiodate (100 ml) for 3 days at 10°. After deionization, the solution was treated with potassium borohydride (0.2 g) for 24 h, deionized again, and evaporated. The residue was hydrolysed with N sulphuric acid for 7 h to yield a mixture of threitol and glycerol which was then examined by paper chromatography in solvent *C*.

Preparation and analysis of the methylated galactan **1**. — Galactan **1** (0.66 g) was completely dissolved in methyl sulphoxide by stirring overnight. The solution obtained was treated for 6 h at 20° in an atmosphere of nitrogen with a solution of the methylsulphinyl carbanion prepared from sodium hydride (0.33 g) and methyl sulphoxide (9.9 ml), as described earlier[12]. Methyl iodide (5 ml) was added, and the reaction mixture was stirred in a nitrogen stream overnight. Recovery of the product (0.46 g) was achieved in the usual manner[13], and this was remethylated in a similar way to afford the methylated galactan **1** (0.2 g) which had 41% of methoxyl groups. Its i.r. spectrum showed the absence of free hydroxyl groups.

The methylated galactan **1** (0.2 g) was dissolved in cold 72% sulphuric acid (0.1 ml) and kept for 1 h at room temperature. The reaction mixture was diluted with water (0.8 ml) and heated in a sealed tube for 4.5 h at 100°. After neutralization with barium carbonate, the solution was concentrated to a syrup (0.175 g), an aliquot of which was diluted with chloroform and examined by t.l.c. and paper chromatography in solvent *B*. The residual syrup (0.17 g) was transferred to a column (2.1 × 31.7 cm) of silica gel and eluted with chloroform and then by a gradient of ethanol in chloroform to give fractions (4 ml each) which were examined by t.l.c. Three combined fractions were obtained as follows: a mixture (4 mg) of 1,2,3,5,6-penta-*O*-methylgalactitol and 2,3,4,6-tetra-*O*-methyl-D-galactose; 2,3,6-tri-*O*-methyl-D-galactose (100 mg); and a mixture (29 mg) of the previous sugar and, probably, the methyl ester of 2,3-di-*O*-methyl-D-galacturonic acid (trace only). Further separation and purification was achieved by preparative, paper chromatography to give 2,3,4,6-tetra-*O*-methyl-D-galactose (25 mg), $[\alpha]_D^{20}$ +117° (water), which was converted into the "anilide", m.p. and mixed m.p. 188°; and 2,3,6-tri-*O*-methyl-D-galactose (80 mg), $[\alpha]_D^{20}$ +80° (water), which was oxidised with bromine to give 2,3,6-tri-*O*-methyl-D-galactono-1,4-lactone, m.p. and mixed m.p. 99.3°.

Methanolysis of the methylated galactan **1** (0.05 g) was achieved by treatment with a mixture of 72% aqueous perchloric acid and methanol (1:10) for 3 h at 100° in a sealed tube. After neutralization and evaporation, the residual syrup was subjected to analysis by g.l.c.

Enzymic digestion of the pectin. — An aqueous extract (1 litre of 1% solution) of roots of *Panax ginseng* was incubated with commercial pectinase (0.3 g) for 96 h at

39–40°, filtered, and subjected to amylolysis as described previously[1]. The solution obtained was deproteinised by the Sevag procedure[14] and poured into methanol to furnish a degraded, pectic polysaccharide, $[\alpha]_D^{20}$ +84° (water) (Found: uronic acid, 4.95; ash, 10.35%).

The polysaccharide material (50 mg) was completely hydrolysed with 2N sulphuric acid, yielding galactose, arabinose, xylose, rhamnose, and small proportions of galacturonic acid and an unidentified sugar having the highest relative mobility on thin-layer and paper (solvent *A*) chromatograms.

Partial hydrolysis of the degraded, pectic polysaccharide. — The material (4 g) in water (300 ml) was hydrolysed with 2N sulphuric acid (30 ml) for 1 h at 100°. The mixture was cooled and poured into ethanol to yield a precipitate of galactan **2** (0.6 g) and a solute of heteropolysaccharide (0.7 g) which was precipitated with acetone. The product was completely hydrolysed with 2N sulphuric acid to afford galacturonic acid, galactose, arabinose, xylose, rhamnose, and an unidentified sugar, as shown by t.l.c. and paper chromatography (solvent *A*).

Preparation and analysis of the methylated galactan **2**. — The galactan **2** (0.6 g) was methylated twice, as described above, to yield the fully methylated product (0.114 g) which contained 33.69% of methoxyl group and contained no hydroxyl groups as indicated by the i.r.-spectrum. Hydrolysis of the material was carried out as above, and the resulting mixture of methylated sugars was examined by t.l.c. and paper chromatography (solvent *B*). The mixture of corresponding methyl glycosides was examined by g.l.c. and t.l.c.

Partial hydrolysis of the heteropolysaccharide. — The polysaccharide (1 g) was hydrolysed with 2N sulphuric acid (25 ml) for 1 h at 100°. The hydrolysate was neutralised with barium carbonate, filtered, concentrated to 6 ml, treated with Amberlite IR-120 (H^+) resin, and transferred to a column with DEAE-Sephadex (HCO_3^-). The column was washed with water until the effluent gave a negative phenol–sulphuric acid test. This procedure yielded two neutral oligosaccharides which were separated by preparative chromatography to afford galactobiose (3 mg) and galactotriose (4 mg). Further gradient elution with aqueous formic acid furnished two acidic oligosaccharides (10.7 and 6.6 mg), each of which, on hydrolysis, yielded D-galacturonic acid and rhamnose, whilst the latter saccharide also afforded xylose.

RESULTS AND DISCUSSION

Attempts to fractionate the pectin by using ethanol, Cetavlon, precipitation with calcium chloride, and formation of cupric complexes were unsuccessful. However, by chromatography on DEAE-cellulose, the pectin was separated into two fractions that were acidic polysaccharides having the same qualitative monosaccharide composition. Chromatography on DEAE-cellulose could not be used for separation of the pectin on a large scale; considerable loss of product occurred during the process, and the alkali used for elution degraded the pectin[18]. On the above basis, the non-fractionated pectin was selected for structural studies. The pectin consumed 0.76 mol

of periodate per sugar moiety, and when the product was reduced with potassium borohydride and then hydrolysed, galactose and small proportions of arabinose and galacturonic acid were produced, suggesting the presence of (1→3)-linked sugar residues and branch points.

It is known that the methylation of polysaccharides containing a high proportion of uronic acid residues is often difficult to achieve and may be accompanied by some degradation[15]. Therefore, the pectin molecule was subjected to partial hydrolysis. A galacturonan was the first fragment obtained on hydrolysis of the pectin with 0.9% sulphuric acid for 3 h. The polysaccharide was converted, by esterification with subsequent reduction[16], into a corresponding galactan in order to facilitate methylation studies. The galactan **1** thus obtained was permethylated according to the method of Hakomori[13] to give a product having a methoxyl content of 41%. Acid hydrolysis gave 2,3,6-tri-(mainly), 2,3,4,6- and 2,3,5,6-(trace)-tetra-*O*-methyl-D-galactose, 1,2,3,5,6-penta-*O*-methylgalactitol, and an unidentified sugar, possibly the methyl ester of 2,3-di-*O*-methyl-D-galacturonic acid. D-Galactose derivatives having a lower extent of methylation were not detected, indicating the absence of branch points in the polygalacturonan chain. It should be noted that 2,3,6-tri-*O*-methyl-D-galactosides were present in the methanolysate as pyranose and furanose forms in the molar ratio *ca.* 3:2 (as shown by g.l.c.). The above data demonstrated that galactan **1** contained a linear chain having (1→4)- and, possibly, (1→5)-linked D-galactose residues. Therefore, the galacturonan had a similar structure. However, there are indications that a furanose form of D-galactose appeared on esterification and subsequent reduction of a polygalacturonan[17], or it might be expected to arise under the conditions used in methanolysis of the methylated galactan. This phenomenon requires more-detailed investigation.

The above structural features of galactan **1** were confirmed by periodate oxidation followed by reduction with borohydride and hydrolysis, yielding threitol and glycerol. The high, positive, specific rotation of the galacturonan demonstrated the predominant α-D configuration of the glycosidic bonds.

A degraded, pectic polysaccharide was obtained by pectinase digestion of the pectin. The material contained D-galactose, D-galacturonic acid (small proportion only), L-arabinose, D-xylose, L-rhamnose, and an unidentified sugar (traces) having the highest mobility on chromatograms. Molecular-sieve chromatography of the degraded, pectic polysaccharide showed a wide distribution with regard to molecular size. Trace amounts of the degraded, pectic polysaccharide were eluted from DEAE-cellulose with sodium phosphate. The main polysaccharide material appeared in effluents having higher pH-values, when elution with alkali was effected. These data demonstrated the acidic character of the polysaccharide[4] and suggest a possible occurrence of bonds between acidic and neutral fragments of the pectin.

Mild hydrolysis of the above polysaccharide with 0.9% sulphuric acid for 45 min resulted in the complete liberation of arabinose, suggesting that residues of this sugar were probably present in the furanose form in the exterior chains. The degraded pectic polysaccharide was subjected to partial hydrolysis with acid to afford

galactan **2** (15.5%) and an acidic heteropolysaccharide (17.5%). The latter consisted of residues of D-galacturonic acid, D-galactose, D-xylose, L-rhamnose, and the unidentified sugar mentioned above. The galactan **2** was permethylated and hydrolysed to yield 2,3,4,6-tetra, and 2,3,4-, 2,3,6-(traces only) and 2,4,6-tri-, and 2,3-di-*O*-methyl-D-galactose, as shown by paper, thin-layer, and gas–liquid chromatography. These data indicate a branched structure for galactan **2** containing (1→6)- and (1→3)-linked D-galactose residues. On partial hydrolysis, the heteropolysaccharide yielded a mixture of mono- and oligo-saccharides, which were fractionated on DEAE-Sephadex into acid and neutral fractions. Further separation was carried out by chromatography on filter sheets and gave a galactobiose and galactotriose, as well as two acidic oligosaccharides, respectively containing galacturonic acid and rhamnose, and galacturonic acid, rhamnose, and xylose.

A more-detailed study of these oligosaccharides was not carried out because of the small amounts available.

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REACTIONS OF GLYCOSYLAMINES WITH β-DIKETONES AND WITH BENZOYLACETALDEHYDE*

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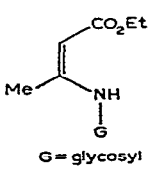
(Received September 5th, 1968)

ABSTRACT

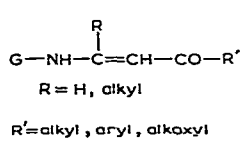
The glycosylamines of the common aldohexoses react with β-diketones and with benzoylacetaldehyde yielding *N*-glycosyl derivatives of β-amino α,β-unsaturated ketones. The structures of these substances are discussed on the basis of their chemical and spectroscopic properties.

INTRODUCTION

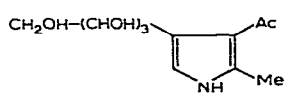
Previous work[1] has shown that glycosylamines react with ethyl acetoacetate to yield ethyl 3-(glycosylamino)crotonates (**1**). Because the *N*-glycosyl derivatives of β-amino α,β-unsaturated ketones and esters (**2**) may be of interest as intermediates in the synthesis of nucleosides and related substances, we have investigated their properties and methods of preparation. In this paper, we describe several ketones of the type **2** obtained by reaction of β-diketones and benzoylacetaldehyde with aldosylamines. The reaction of 2,4-pentanedione with glycosylamines has been previously studied[2], and, under the conditions used, resulted in the formation of (tetrahydroxybutyl)pyrroles (*e.g.*, compound **3**), presumably *via* intermediates of



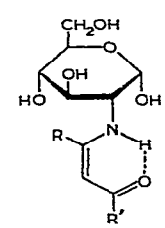
1



2



3



4 R = R' = Me
5 R = H, R' = Ph
6 R = Me, R' = Ph

*Presented in part at the 12th Biennial Meeting of the Real Sociedad Española de Física y Química, Salamanca, June 1965; A. Gómez Sánchez, J. Velasco del Pino, and U. Scheidegger, *Anales Real Soc. Españ. Fis. Quim.* (Madrid), 62-B (1966) 487.

TABLE I

N-GLYCOSYL DERIVATIVES OF β-AMINO α,β-UNSATURATED KETONES **7a–16a** AND THEIR *O*-ACETYL DERIVATIVES **7b–16b**

Substance	*M.p.*, ° (*crystallization solvent*)	$[\alpha]_{5461}$ (*degrees*)	*Yield* (%)	*Formula*	*Calc.*			*Found*		
					C	*H*	*N*	*C*	*H*	*N*
7a	[a]									
7b	192–195 (propan-2-ol)	− 41.6 (chloroform)		$C_{23}H_{27}NO_{10}$	57.85	5.7	2.9	58.2	5.8	2.7
8a	175–177 (ethanol–ethyl acetate)	−100.2 (pyridine)	34	$C_{16}H_{21}NO_6$	59.4	6.55	4.3	59.2	6.4	4.7
8b	177–178 (propan-2-ol)	− 28.5 (chloroform)	78	$C_{24}H_{29}NO_{10}$	58.65	5.95	2.85	58.8	6.0	2.9
9a	177–179 (ethanol)	−105.8 (pyridine)	80	$C_{15}H_{19}NO_6$	58.2	6.2	4.5	58.3	6.2	4.1
9b	124–126 (ether–light petroleum)	0.0 (chloroform)	44	$C_{23}H_{27}NO_{10}$	57.85	5.7	2.9	57.9	5.5	3.0
10a	195–196 (ethanol)	−179.3 (pyridine)	38	$C_{16}H_{21}NO_6$	59.4	6.55	4.3	59.4	6.7	4.3
10b	106–108 (ether–light petroleum)	− 11.1 (chloroform)	45	$C_{24}H_{29}NO_{10}$	58.65	5.95	2.85	58.5	6.0	2.9
11a	223–225 (ethanol)	−158.0[b] (ethanol)	5	$C_{11}H_{19}NO_6$	50.6	7.3	5.4	50.8	7.0	5.2
11b	104–105 (ethanol–water)	− 16.0[b] (ethanol)	64	$C_{19}H_{27}NO_{10}$	53.1	6.3	3.5	53.1	6.4	3.7
12a	188–190 (ethanol–water)	−203.7 (ethanol)	82	$C_{15}H_{19}NO_6$	58.2	6.2	4.5	58.4	6.4	4.35
12b	144–145 (carbon tetrachloride)	− 32.0 (chloroform)	85	$C_{23}H_{27}NO_{10}$	57.85	5.7	2.9	57.6	5.9	3.2
13a	214–215 (ethanol)	−194.2 (pyridine)	9	$C_{16}H_{21}NO_6$	59.4	6.55	4.3	59.6	6.7	4.4
13b	168–169 (propan-2-ol)	0.0 (chloroform)	75	$C_{24}H_{29}NO_{10}$	58.65	5.95	2.85	58.5	5.7	3.0
14a	213–214 (methanol)	−164.0[b] (ethanol)	11	$C_{11}H_{19}NO_5$	53.8	7.8	5.7	54.0	7.9	5.7
14b	180–182 (ethanol–water)	− 40.0[b] (ethanol)	71	$C_{17}H_{25}NO_8$	55.0	6.8	3.8	55.3	6.8	3.7
15a	188–189 (ethanol)	−153.0 (ethanol)	53	$C_{15}H_{19}NO_5 \cdot H_2O$	57.9	6.8	4.5	58.15	6.65	4.6
15b	178–179 (propan-2-ol)	−103.2 (carbon tetrachloride)	89	$C_{21}H_{25}NO_8$	60.1	6.0	3.3	60.1	5.8	3.6
16a	220–221 (ethanol)	−140.3 (pyridine)	36	$C_{16}H_{21}NO_5 \cdot H_2O$	59.1	7.2	4.3	59.2	7.4	4.3
16b	194–196 (propan-2-ol)	− 78.4 (carbon tetrachloride)	93	$C_{22}H_{27}NO_8$	60.95	6.3	3.2	60.9	6.3	3.2

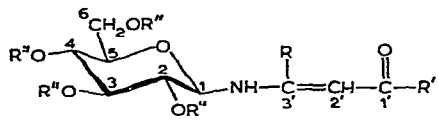
[a]Substance obtained as a syrup. [b]$[\alpha]_D$ value.

structure 2 (R = R′ = Me). It is also known[3] that 2-amino-2-deoxy-D-glucose reacts with 2,4-pentanedione, 1-phenyl-1,3-butanedione, and benzoylacetaldehyde, yielding derivatives **4–6**.

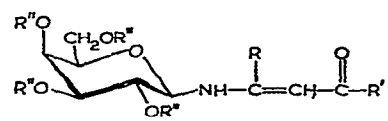
RESULTS AND DISCUSSION

The reactions of the glycosylamines of the common aldohexoses (D-glucose, D-galactose, D-mannose, and L-rhamnose) with β-diketones (2,4-pentanedione, 1-phenyl-1,3-butanedione) and with benzoylacetaldehyde took place in methanol at room temperature yielding *N*-glycosyl derivatives of β-amino α,β-unsaturated ketones **7a–16a**. Reactions with 1-phenyl-1,3-butanedione were better carried out in methanol containing acetic acid, as otherwise the corresponding products were obtained in admixture with other unidentified substances. The physical constants and yields of the products are indicated in Table I. The reactions of β-D-glucopyranosylamine and β-D-galactopyranosylamine with 2,4-pentanedione gave syrups which could neither be crystallized nor converted into crystalline derivatives. The condensate **7a** of β-D-glucopyranosylamine and benzoylacetaldehyde was also obtained as a syrup which was easily transformed into a crystalline tetra-*O*-acetyl derivative **7b**. Compound **14a** was identical with that obtained directly in the reaction of L-rhamnose with 2,4-pentanedione and ammonia[4].

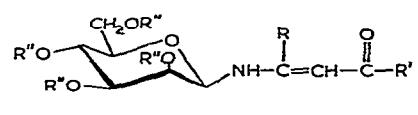
Evidence for the structures of these substances is presented in the following discussion of their properties. They gave positive Fehling's and ferric chloride tests,



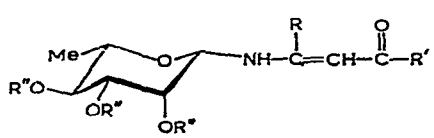
	R	R′	R″
7a	H	Ph	H
7b	H	Ph	Ac
8a	Me	Ph	H
8a	Me	Ph	Ac



	R	R′	R″
9a	H	Ph	H
9b	H	Ph	Ac
10a	Me	Ph	H
10b	Me	Ph	Ac



	R	R′	R″
11a	Me	Me	H
11b	Me	Me	Ac
12a	H	Ph	H
12b	H	Ph	Ac
13a	Me	Ph	H
13b	Me	Ph	Ac



	R	R′	R″
14a	Me	Me	H
14b	Me	Me	Ac
15a	H	Ph	H
15b	H	Ph	Ac
16a	Me	Ph	H
16b	Me	Ph	Ac

presumably because of their hydrolysis to the parent β-dicarbonyl compound and sugar. Hydrolysis also took place during the chromatography, on paper, of analytically

pure samples of the 2,4-pentanedione derivatives **11a** and **14a**, and of the condensate of 1-phenyl-1,3-butanedione with β-D-glucopyranosylamine (**8a**), since spots appeared corresponding to the parent glycoses and glycosylamines in addition to those of the condensates (see Table II). Similar observations have been made[1a] on ethyl 3-(glycosylamino)crotonates (**1**) and on simpler glycosylamines[5]. Further evidence of the formation of free glycoses and β-dicarbonyl compounds in the hydrolysis of the substances under discussion was obtained for 3-(β-D-glucopyranosylamino)-1-phenyl-2-buten-1-one (**8a**); treatment of this compound with 0.2N hydrochloric acid gave 1-phenyl-1,3-butanedione and D-glucose (characterized as *N*-*p*-nitrophenyl-β-D-glucopyranosylamine). However, the *N*-glycosyl derivatives of β-amino α,β-unsaturated ketones seemed to be more resistant to hydrolytic cleavage than ethyl 3-(glycosylamino)crotonates; for example, the optical rotation of an aqueous solution of 4-(β-D-mannopyranosylamino)-3-penten-2-one (**11a**) changed from $+251 \rightarrow +120°$ in two months without having reached equilibrium; paper chromatography then showed the presence of D-mannose and unchanged compound **11a**. Under the same conditions, ethyl 3-(β-D-mannopyranosylamino)crotonate was completely hydrolyzed in five days.

TABLE II

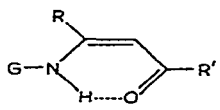
CHROMATOGRAPHIC MOBILITIES[a] OF COMPOUNDS **7a–16a** AND REFERENCE SUBSTANCES

Substance (formula)	R_F			
3-(β-D-Glucopyranosylamino)-1-phenyl-2-buten-1-one (**8a**)	0.06,	0.12,	0.15,	0.67
β-D-Glucopyranosylamine	0.07,	0.13,	0.16	
D-Glucose			0.16	
3-(β-D-Galactopyranosylamino)-1-phenyl-2-propen-1-one (**9a**)				0.59
3-(β-D-Galactopyranosylamino)-1-phenyl-2-buten-1-one (**10a**)				0.68
4-(β-D-Mannopyranosylamino)-3-penten-2-one (**11a**)			0.21,	0.40
3-(β-D-Mannopyranosylamino)-1-phenyl-2-propen-1-one (**12a**)				0.60
3-(β-D-Mannopyranosylamino)-1-phenyl-2-buten-1-one (**13a**)				0.66
D-Mannose			0.21	
4-(β-L-Rhamnopyranosylamino)-3-penten-2-one (**14a**)			0.30	0.60
3-(β-L-Rhamnopyranosylamino)-1-phenyl-2-propen-1-one (**15a**)				0.73
3-(β-L-Rhamnopyranosylamino)-1-phenyl-2-buten-1-one (**16a**)				0.73
L-Rhamnose			0.30	

[a]On Whatman No. 1 paper with butyl alcohol–water–ammonia (40:10:49:1, organic phase) as solvent; R_F measured at 20°.

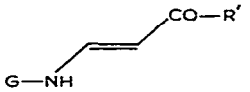
Treatment of compounds **7a–16a** with acetic anhydride in pyridine at 0° gave the corresponding acetates **7b–16b**. The physical constants of these substances have been included in Table I. Catalytic (sodium methoxide) deacetylation of 1-phenyl-3-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamino)-2-buten-1-one (**8b**) regenerated the parent compound **8a**; hydrolysis of **8b** with dilute formic acid[6] gave 1-phenyl-1,3-butanedione and 2,3,4,6-tetra-*O*-acetyl-D-glucose.

The u.v. spectra of compounds **8a–16a** (Table III) were similar to those[3] of the 2-amino-2-deoxy-D-glucose derivatives[3] **4–6**. The i.r. spectra of compounds **8a–16a** and those of the acetates **7b–16b** (Table III) showed bands at 1640–1600 (ketone C=O), 1585–1575, and 1560–1530 cm^{-1} typical of intramolecularly bonded β-amino α,β-unsaturated ketones[3b,c7]. The large shift to low frequencies of the carbonyl band of these compounds is considered to be a consequence of the highly pronounced mesomeric character of the enamino ketone system and of the strong, intramolecular hydrogen bond. For the same reasons, the N-H stretching bands of the acetates were very broad and displaced to low frequencies. It was then concluded that, in the solvents used, compounds **7–16** were completely, or to a large extent, in the chelated form **17**.



G=glycosyl

17



18 G=β-D-galactopyranosyl
19 G=β-D-mannopyranosyl

The p.m.r. spectra of compounds **7–16** confirmed their structures and gave an indication of their anomeric configurations. Data from the spectra of the acetates of the condensates of benzoylacetaldehyde, namely, compounds **7b**, **9b**, **12b**, and **15b**, are indicated in Table IV. The absorptions of the enamine portion of these substances were very similar to those of the intramolecularly bonded *cis* forms of the enamines derived from benzoylacetaldehyde and simple amines[8] and to that[3c] of the tetra-*O*-acetyl derivative of compound **5**. The chelated *cis* structures were derived from the positions of the N-H signals at very low field and the magnitude of the vicinal coupling constants, $J_{2',3'}$, between the two olefinic protons. The protons of the sugar moiety of these substances, as well as those of compounds discussed later, were identified by double-resonance experiments and/or by consideration of the chemical shifts and the coupling constants. All of these chemical shifts and coupling constants observed were consistent with the compounds, having pyranose rings. The assignment of the β-D-anomeric configuration was straightforward in the D-glucose and D-galactose derivatives, compounds **7b** and **9b**, by considering the values of $J_{1,2}$. In the case of the D-mannose and L-rhamnose derivatives (**12b**) and (**15b**), respectively, the assignment of the anomeric configuration was tentatively made by comparing the chemical shifts for H-1 with those for the same proton in compounds **7a** and **9b** having the D-*gluco* and D-*galacto* configuration. The paramagnetic shift of 0.20–0.29 p.p.m. observed thereby was ascribed to the deshielding effect produced on an axial H-1 by the change of the acetoxyl group on C-2 from the equatorial to the axial position. Similar paramagnetic shifts have been observed before in acetylated aldopyranoses[9a,b] in penta-acetylglycopyranosylamines[9c], and in the *O*-acetyl derivatives of ethyl 3-(glycosylamino)crotonates[1b]. Substances **12b** and **15b** would then have H-1 in an axial position and, therefore, β-D and β-L configurations, respectively.

The p.m.r. spectra of 3-(glycosylamino-1-phenyl-2-propen-1-ones (**9a**, **12a**, and **15a**) (Table V) were taken in methyl sulphoxide-d_6 in order to prevent effects due to hydrolysis and/or mutarotation[1b,10]. Under these conditions, the D-galactose

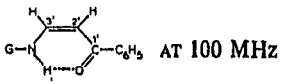
TABLE III

U.V. AND I.R. ABSORPTION[a] OF COMPOUNDS 7–16

Substance	U.v. absorption[b]		I.r. absorption (cm^{-1})		
	λ_{max} nm	log ε	Phase	O–H and/or N–H	H–N–C=C–C=O
7b			Cf	3250w,b	1636, 1585, 1559m
8a	245	3.86	N	3320b	1595b[c], 1576, 1538b
8b			Cf	3200vw,b	1620, 1587, 1559
9a	247	3.84	N	3550, 3280b, 3198b	1622, 1580, 1530
9b			Cf	3240vw,b	1638, 1585, 1559m
10a	244	3.88	N	3401, 3172b	1600b[c], 1580, 1532
10b			Cf	3200w,b	1618, 1583, 1556
11a	303	4.28	N	3448m,b, 3135m	1613, 1547b
11b			C	3200w,b	1629, 1590, 1502m
12a	248	3.83	N	3540m, 3430, 3305b, 3250b	1630b, 1578, 1543b
12b			Cf	3250vw,b	1639b, 1588, 1560m
13a	244	3.84	N	3350b, 3281b	1600b[c], 1574, 1525b
13b			Cf	3180w,b	1619, 1583, 1559b
14a	303	4.37	N	3440b, 3180m	1614, 1550, 1530m
14b			C	3185w,b	1623, 1577, 1502m
15a	248	3.85	N	3350b, 3274b, 3180b	1632, 1580, 1532b
15b			Cf	3240vw,b	1636, 1584, 1556
16a	244	3.84	N	3344b, 3170b	1601b[c], 1576, 1533b
16b			Cf	3090vw,b	1608b, 1584, 1558m

[a]Abbreviations: N, solid in Nujol mull; C, carbon tetrachloride solution; Cf, chloroform solution; m, medium; w, weak; vw, very weak; and b, broad. When no indication of the intensity is given, it is implied that a strong band was observed. [b]In ethanol. [c]Band including a stretching vibration of the benzene ring.

TABLE IV

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) FOR *O*-ACETYL DERIVATIVES OF COMPOUNDS  AT 100 MHz IN CHLOROFORM-*d*

Substance	*N-H*	*H-2'*	*H-3'*	*Aromatic protons*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*OAc*
7b	10.23q[a] $J_{NH,3'}$ 12.0 $J_{NH,1}$ 9.1	5.87d $J_{2',3'}$ 8.2	6.91q	~7.4m[b] ~7.85m[c]	4.50t $J_{1,2}$ 9.0	4.90–5.40m			3.76m $J_{4,5}$ 9.5 $J_{5,6}$ 4.5 $J_{5,6'}$ 2.5	4.24q $J_{6,6'}$ −12.4	4.10q	2.01[d], 2.06
9b	10.31q $J_{NH,3'}$ 12.0 $J_{NH,1}$ 9.0	5.89d $J_{2',3'}$ 8.0	6.92q	~7.4m[b] ~7.88m[c]	4.46t $J_{1,2}$ 9.0	5.34q $J_{2,3}$ 10.3	5.10q $J_{3,4}$ 3.2	5.43 $J_{4,5} \simeq 1$	3.90–4.15m			1.98, 2.01 2.02[e], 2.16
12b	10.43q $J_{NH,3'}$ 12.0 $J_{NH,1}$ 9.6	5.85d $J_{2',3'}$ 8.0	6.92q	~7.4m[b] ~7.85m[c]	4.75q $J_{1,2}$ 1.6	5.49q $J_{2,3}$ 3.2	5.10q $J_{3,4}$ 10.0	5.25t $J_{4,5}$ 9.0	3.72m $J_{5,6}$ 5.3 $J_{5,6'}$ 3.0	4.26q $J_{6,6'}$ −12.4	4.12q	1.99, 2.04 2.07, 2.34
15b	10.42q $J_{NH,3'}$ 11.8 $J_{NH,1}$ 9.3	5.83d $J_{2',3'}$ 8.0	6.93q	~7.4m[b] ~7.85m[c]	4.70q $J_{1,2}$ 1.5	5.47t $J_{2,3}$ 2.5			3.58m $J_{5,6}$ 6.2		1.25d[f]	1.99, 2.05 2.33

[a]Signal multiplicities are indicated as following: d, doublet; t, triplet; q, quartet; and m, multiplet. The absence of any indication implies that a singlet was observed. [b]Complex signal of intensity three. [c]Complex signal of intensity two. [d]Signal of intensity nine. [e]Intensity six. [f]Signal due to *C*-5 methyl group.

TABLE V

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF COMPOUNDS G–NH–CH(3′)=CH(2′)–CO(1′)–Ph AT 100 MHz IN METHYL SULPHOXIDE-d_6

Substance	*N-H* cis	trans	*H-2′* cis	trans	*H-3′* cis	trans	*Aromatic protons*	*H-1*	cis-trans *ratio*
9a	10.29q[a]	[b]	5.85d	6.14d	7.27q	[b]	~7.47m[c]	4.31t	2:1
	$J_{NH,3'}$ 12.5		$J_{2',3'}$ 8.0	$J_{2',3'}$ 12.4			~7.85m[d]	$J_{1,2}$ 8.0	
	$J_{NH,1}$ 8.5								
12a	10.37q	[b]	5.82d	6.21d	7.24q	[b]	~7.47m[c]	4.67q	4:1
	$J_{NH,3'}$ 12.6		$J_{2',3'}$ 8.0	$J_{2',3'}$ 11.6			~7.85m[d]	$J_{1,2} \simeq 1.0$	
	$J_{NH,1}$ 9.4								
15a	10.37q		5.82		7.21q		~7.45m[c]	4.63q	
	$J_{NH,3'}$ 13.0		$J_{2',3'}$ 8.0				~7.85m[d]	$J_{1,2} \sim 1.0$	
	$J_{NH,1}$ 9.5								

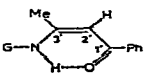
[a]Multiplicities are indicated as following: d, doublet; t, triplet; q, quartet; and m, multiplet. [b]Not located. [c]Complex signal of intensity three. [d]Complex signal of intensity two.

and D-mannose derivatives, (**9a**) and (**12a**), existed as a mixture of the *cis* form (type **17**) and the corresponding *trans* form (**18** and **19**, respectively). The existence of the two isomeric forms of the 3-(β-D-galactopyranosylamino)-1-phenyl-2-propen-1-one (**9a**) was deduced from the presence in the spectrum (Table V) of the two doublets at δ 5.85 ($J_{2',3'}$ 8.0 Hz) and δ 6.14 ($J_{2',3'}$ 12.4 Hz) which, based on the coupling constants, were assigned to H-2′ of the *cis* and *trans* forms, respectively. The quartet at δ 7.27 ($J_{NH,3'}$ 12.5 Hz; $J_{2',3'}$ 8.0 Hz) was assigned to H-3′ of the *cis* form; the corresponding signal of the *trans* form was not observed and must be hidden under the multiplet of the aromatic protons (δ 7.4–8.0). The quartet centered at δ 10.29 ($J_{NH,3'}$ 12.5 Hz; $J_{NH,1}$ 8.5 Hz) was ascribed to the N-H of the *cis* form, and the triplet ($J_{NH,1}$ 8.5 Hz; $J_{1,2}$ 8.0 Hz) at δ 4.31 to H-1. These assignments were confirmed by irradiation of the N-H quartet, causing the collapse of the H-1 and *cis* H-3′ signals. Furthermore, treatment of the sample with deuterium oxide changed the *cis* H-3′ quartet into a doublet ($J_{2',3'}$) and the H-1 triplet into a doublet ($J_{1,2}$). The *cis-trans* ratio (2:1) for compound **9a** was obtained from the integral values of the H-2′ doublets, and the magnitude of $J_{1,2}$ proved the β-D-anomeric configuration. Similar observations were made in the spectrum of 3-(β-D-mannopyranosylamino)-1-phenyl-2-propen-1-one (**12a**) (Table V) which was a mixture of the *cis* and *trans* forms in the approximate ratio of 4:1. A different picture, however, was shown by the p.m.r. spectrum of the L-rhamnose analogue (**15a**) which, under the conditions observed, existed in the *cis* form only. The anomeric configurations of the two last compounds were tentatively assigned by considering again the δ-values of the H-1 protons; they showed paramagnetic shifts of approximately 0.35 p.p.m. relative to H-1 in the D-galactose derivative **9b**. This paramagnetic effect is of the same order of magnitude as that produced on an axial H-1 of glycopyranoses by changing the hydroxyl group on C-2 from an equatorial to an axial disposition[9a,11]. Consequently, H-1 in compounds **12a** and **15a** should be axial, and have the β-D- and β-L-anomeric configurations, respectively. Assignments on the same basis have been previously made[1b] in ethyl 3-(glycosylamino)crotonates.

Data of the p.m.r. spectra of the condensates of 1-phenyl-1,3-butanedione and those of their corresponding *O*-acetyl derivatives are presented in Tables VI and VII. The chelated structures (**17**) of these substances were deduced from the presence of the N-H signals at very low fields and from the similarity of these spectra with those of the intramolecularly bonded forms of enamines obtained in the reactions of 1-phenyl-1,3-butanedione with simple amines[12] and with 2-amino-2-deoxy-D-glucose[3c]. The value for $J_{1,2}$ proved the β-D-anomeric configuration in the derivatives of D-glucose (compounds **8a** and **8b**) and D-galactose (compounds **10a** and **10b**). The H-1 protons in the condensates of D-mannosylamine and L-rhamnosylamine (**13a** and **16a**, respectively) showed paramagnetic shifts of approximately 0.30 p.p.m. relative to H-1 in compound **8a** having the D-*gluco* configuration; similarly, the H-1 signals in the corresponding *O*-acetyl derivatives (**13b** and **16b**) were shifted approximately 0.15 p.p.m. to lower field compared to compound **8b** having the D-*gluco* configuration. These paramagnetic shifts are those expected[1b,9,11] for an

axial disposition of H-1 in all of these compounds. Therefore, the derivatives of D-mannose (**13a** and **13b**) were tentatively assigned the β-D-anomeric configuration and those of L-rhamnose (**16a** and **16b**) the β-L-anomeric configuration.

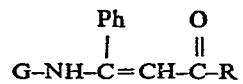
TABLE VI

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF COMPOUNDS  AT 100 MHz IN METHYL SULPHOXIDE-d_6

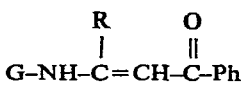
Substance	*N-H*	*H-2'*	*Me-3'*	*Aromatic protons*	*H-1*
8a	11.27d[a]	5.84	2.18	~7.44m[b]	4.62t
	$J_{NH,1}$ 8.2			~7.84m[c]	$J_{1,2}$ 8.0
10a	11.28d	5.84	2.13	~7.43m[b]	4.56t
	$J_{NH,1}$ 8.5			~7.85m[c]	$J_{1,2}$ 7.4
13a	11.34d	5.82	2.16	~7.41m[b]	4.90q
	$J_{NH,1}$ 9.0			~7.82m[c]	$J_{1,2}$ ~1.0
16a	11.27d	5.81	2.13	~7.42m[b]	4.87q
	$J_{NH,1}$ 9.0			~7.82m[c]	$J_{1,2}$ ~1.0

[a]Signal multiplicities are indicated as following: d, doublet; t, triplet; q, quartet; and m, multiplet. The absence of any indication implies that a singlet was observed. [b]Complex signal of intensity three. [c]Complex signal of intensity two.

The reaction between a primary amine and an unsymmetrical β-dicarbonyl compound can, in principle, give rise to two isomeric enamines. Thus, the condensates of glycosylamines with benzoylacetaldehyde might have structure **20** instead of the structure of type **22** considered above. The former possibility, however, can be



20 R = H **21** R = Me

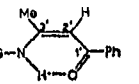


22 R = H

ruled out purely on the basis of the p.m.r. spectra already discussed (Tables IV and V). They clearly indicate the formation of di-substituted olefins rather than of α,β-unsaturated aldehydes. This is further confirmed by the similarity of these spectra with those corresponding to the condensates of benzoylacetaldehyde with primary amines [8] and with 2-amino-2-deoxy-D-glucose[3c]. It has been well established that these substances react with the carbonyl group of benzoylacetaldehyde and of 1-phenyl-1,3-butanedione which is further away from the phenyl group. Similar arguments precluded structure **21** for the condensation products of glycosylamines and 1-phenyl-1,3-butanedione.

The p.m.r. spectra of the enamine portion of the 2,4-pentanedione derivatives **11a**, **11b**, and **14b** (Table VIII) were very similar to those of the chelated form of

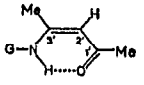
TABLE VII

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF *O*-ACETYL DERIVATIVES OF COMPOUNDS  AT 100 MHz IN CHLOROFORM-*d*

Substance	*N-H*	*H-2'*	*:C-Me*	*Aromatic protons*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*OAc*
8b	11.19d[a]	5.82	2.12	~7.40m[b]	4.87t		5.05–5.30m		3.75m	4.22q	4.10	2.02 2.06[d]
	$J_{NH,1}$ 8.3			~7.85m[c]	$J_{1,2}$ 8.5				$J_{5,6}$ 4.8	$J_{6,6'}$ −12.4		
									$J_{5,6'}$ 3.0			
10b	11.24d	5.81	2.12	~7.40m[b]	4.82t	5.39t	5.10q	5.41q	3.85–4.15m			1.98, 2.01[e]
	$J_{NH,1}$ 9.0			~7.85m[c]	$J_{1,2}$ 8.8	$J_{2,3}$ 9.5	$J_{3,4}$ 3.0	$J_{4,5}$ ~1				2.15
13b	11.47d	5.77	2.11	~7.40m[b]	5.04q	5.51q	5.11a	5.23t	3.72m	4.24q	4.11q	2.00, 2.04
	$J_{NH,1}$ 9.4			~7.80m[c]	$J_{1,2}$ 1.5	$J_{2,3}$ 3.0	$J_{3,4}$ 10.0	$J_{4,5}$ 9.5	$J_{5,6}$ 5.5	$J_{6,6'}$ −12.4		2.06, 2.41
									$J_{5,6'}$ 3.0			
16b	11.47d	5.75	2.11	~7.40m[b]	5.00q	5.48m			3.58m		1.25d[f]	1.99, 2.04
	$J_{NH,1}$ 9.5			~7.85m[c]	$J_{1,2}$ 1.5				$J_{5,6}$ 6.2			2.40

[a]Signal multiplicities are indicated as following: d, doublet; t, triplet; q, quartet; and m, multiplet. The absence of any indication implies that a singlet was observed. [b]Complex signal of intensity three. [c]Complex signal of intensity two. [d]Signal of intensity nine. [e]Signal of intensity six. [f]Signal due to *C*-5 methyl group.

TABLE VIII

CHEMICAL SHIFTS (δ, p.p.m.) AND COUPLING CONSTANTS (Hz) OF COMPOUNDS  AT 100 MHz

Substance	*Solvent*	*N-H*	*H-2'*	*Me-1', Me-3', and OAc*	*H-1*	*H-2*	*H-5*	*H-6*	*H-6'*
11a	Methyl sulphoxide-d_6	11.69d[a]	5.01	1.88, 1.97	4.75q				
		$J_{NH,1}$ 9.1			$J_{1,2}\sim$1.0				
11b	Chloroform-*d*	10.91d	5.12	2.0[b], 2.02,	4.95q	5.46q	3.70m	4.11q	4.24q
		$J_{NH,1}$ 9.5		2.05, 2.08,	$J_{1,2}$ 1.5	$J_{2,3}$ 3.2	$J_{5,6}$ 5.5	$J_{6,6'}$ −12.0	
				2.35			$J_{5,6}$ 2.8		
14b	Chloroform-*d*	10.86d	5.10	1.98, 1.99,	4.92q	5.43m	3.55m	1.25d[d]	
		$J_{NH,1}$ 9.5		2.04[c], 2.32	$J_{1,2}$ 1.5		$J_{5,6}$ 6.0		

[a]Signal multiplicities are indicated as following: d, doublet; q, quartet; and m, multiplet. The absence of any indication implies that a singlet was observed. [b]Signal of intensity six. [c]Total intensity of the singlets at δ 1.98, 1.99, and 2.04, twelve. [d]*C*-5 methyl signal.

4-(alkylamino)-3-penten-2-one[8,12] and to that[3c] of the tetra-*O*-acetyl derivative of compound **4**. On the other hand, H-1 in compound **11a** had almost the same δ-value as ethyl 3-(β-D-mannopyranosylamino)crotonate[1b]; similarly, H-1 of the *O*-acetyl derivatives **11b** and **14b** had the same δ-values as the *O*-acetyl derivatives of ethyl 3-(β-D-mannopyranosylamino)crotonate and ethyl 3-(β-L-rhamnopyranosylamino)-crotonate[1b]. Compounds **11a**, **11b**, and **14b** were therefore formulated with the chelate structure **17** (R = R′ = Me) and, tentatively, with the β-D- or β-L-anomeric configurations.

The above results and those previously reported[1,3] lead to the conclusion that the reaction of β-dicarbonyl compounds and primary amines is extensible to amino sugars (glycosylamines and 2-amino-2-deoxy-D-glucose). The *N*-glycosyl derivatives of β-amino α,β-unsaturated ketones and esters produced in these reactions tend to adopt the chelate structure **17** which requires that the amino and keto (or ester) groups are in *cis* disposition. Furthermore, the compounds of this kind having the D-*gluco*, D-*galacto*, and D-*manno* configurations are obtained as β-D anomers, and those having the L-*rhamno* configurations as β-L anomers.

EXPERIMENTAL

Melting points are uncorrected. Solutions were evaporated below 40°. Identification of compounds was based on mixed melting points and comparison of i.r. spectra. Paper chromatography was performed on Whatman No. 1 paper, with butyl alcohol–ethanol–water–ammonia (40:10:49:1, organic phase) as the developer and indication with alkaline silver nitrate. Optical rotations at 5461Å were determined with a Bendix–Ericsson Type 143C polarimeter. The u.v. spectra were obtained on a Beckman DU spectrophotometer, and i.r. spectra on a Perkin–Elmer 621 instrument. The p.m.r. spectra were measured on a Varian HA-100 spectrometer, with tetramethylsilane (δ = 0) as the internal standard. The glycosylamines were prepared as described previously[1a]. Physical constants, crystallization solvents, yields, and analytical data of new compounds are indicated in Table I.

Reactions of aldosylamines with benzoylacetaldehyde. — A suspension of 0.01 mole of glycosylamine in methanol (40 ml) containing 0.01 mole of benzoylacetaldehyde was shaken at room temperature until complete dissolution occurred (1–14 h). Evaporation of the solvent left a crystalline residue which, after recrystallization from the appropriate solvent, gave pure 3-(glycosylamino)-1-phenyl-2-propen-1-one (**9a**, **12a**, and **15a**). The derivative **7a** from β-D-glycopyranosylamine was obtained as a syrup that could not be crystallized.

Reactions of aldosylamines with 1-phenyl-1,3-butanedione. — A suspension of 0.01 mole of glycosylamine in methanol (40 ml) containing acetic acid (2 ml) and 0.01 mole of 1-phenyl-1,3-butanedione was shaken at room temperature till dissolution (1–18 h). The crystalline residue left after evaporation of the solvent was recrystallized from the appropriate solvent, yielding 3-(glycosylamino)-1-phenyl-2-buten-1-one (**3a**, **10a**, **13a**, and **16a**).

Reactions of aldosylamines with 2,4-pentanedione. — A suspension of 0.01 mole of D-mannosylamine or L-rhamnosylamine in methanol (20 ml) containing 0.03 mole of 2,4-pentanedione was shaken at room temperature until dissolution. Evaporation of the solvent left a syrup which crystallized upon treatment with ether and then with warm methanol. Several recrystallizations from methanol or ethanol gave the pure product **11a** or **14a**. The reactions with β-D-glucopyranosylamine and β-D-galactopyranosylamine gave syrups that could not be crystallized.

Acetylation of compounds **7a–16a.** — A solution or suspension of the sample (1.0 g) in pyridine (10 ml) was treated with acetic anhydride (5 ml) for 24–48 h at 0° with occasional shaking. The mixture was poured onto ice, and the material that separated was washed thoroughly with water. In most cases, crystalline substances were obtained at this stage and were readily recrystallized in the solvents indicated in Table I. When the product remained syrupy, it was dissolved in chloroform. The solution was washed successively with 0.2N sulphuric acid, saturated, aqueous sodium carbonate, and water, and was then dried ($MgSO_4$). The residue obtained after evaporation was crystallized from the appropriate solvent.

Hydrolysis of 3-(β-D-glucopyranosylamino)-1-phenyl-2-buten-1-one (**8a**). — A solution of compound **8a** (1.0 g) in 0.2N hydrochloric acid (50 ml) was kept for 24 h at room temperature. The crystalline solid that separated was filtered off and, after recrystallization from propan-2-ol, identified as 1-phenyl-1,3-butanedione (0.47 g, 94%). Paper chromatography of the filtrate showed the presence of substances having the same mobilities as di-D-glucosylamine, D-glucosylamine, and D-glucose (main product). Evaporation of this solution gave a syrupy residue. A portion (0.3 g) of this syrup was treated with *p*-nitroaniline (0.5 g) and conc. hydrochloric acid (0.02 ml) in boiling methanol (20 ml). Refrigeration of the reaction mixture gave *N-p*-nitrophenyl-β-D-glucopyranosylamine dihydrate (0.2 g), m.p. 187–189°, identical with an authentic specimen.

Deacetylation of 1-phenyl-3-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamino)-2-buten-1-one (**8b**). — A sample (1.0 g) of compound **8b** was deacetylated by treatment with methanol (10 ml) and 1 ml of a 0.2M solution of sodium methoxide in methanol for 3 h at room temperature. Evaporation of solvent gave a crystalline residue (0.7 g) which, after recrystallization from ethanol–ethyl acetate, gave pure 3-(β-D-glucopyranosylamino)-1-phenyl-2-buten-1-one (**8a**).

Hydrolysis of 1-phenyl-3-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamino)-2-buten-1-one (**8b**). — A solution of compound **8b** (1.0 g) in a mixture of 1% formic acid (50 ml) and ethanol (15 ml) was boiled for 7 h. The solid that separated was filtered off, recrystallized from propan-2-ol, and identified as 1-phenyl-1,3-butanedione (0.2 g, 40%). The filtrate was concentrated, and extracted with chloroform (4 × 5 ml). The combined extracts were washed successively with 0.2N hydrochloric acid, 5% aqueous sodium hydrogen carbonate, 2% aqueous sodium bisulphite, and water (10 ml of each), and dried ($CaCl_2$). The solvent was removed, and the residual syrup was crystallized from chloroform–light petroleum, yielding 2,3,4,6-tetra-*O*-acetyl-D-glucose (0.16 g), m.p. 121–122°, identical with an authentic specimen.

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STUDIES OF 2-AMINO-2-DEOXY-DL-GLYCERALDEHYDE. IDENTIFICATION OF AN *N*-ACETYL DERIVATIVE AMONGST THE DEGRADATION PRODUCTS OF THE OVALBUMIN GLYCOPEPTIDE

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ABSTRACT

Reduction of ethyl 2-amino-3,3-diethoxypropionate with lithium aluminium hydride yields the acetal, $CH_2OH \cdot CHNH_2 \cdot CH(OC_2H_5)_2$ (**1**). The product obtained on hydrolysis of **1** with concentrated hydrochloric acid is stable only in acid solution and behaves as a hydrate or a polymer of $CH_2OH \cdot CH\overset{+}{N}H_3 \cdot CHO\ Cl^-$. It affords a crystalline semicarbazone hydrochloride (**2**), 2,4-dinitrophenylhydrazone hydrochloride (**3**), and dithioacetal oxalate (**4**). Other acyl and alkyl derivatives have been prepared directly from compound **1**. The aldehyde, $CH_2OH.CH(NHCOCH_3).CHO$, which has only been identified in solution, should be formed after the Smith degradation of certain polysaccharides that contain amino sugars. The diethyl acetal **12** and its *O*-methyl derivative **15**, the 2,4-dinitrophenylhydrazone **13**, and the ethylene dithioacetal **14** of this aldehyde have been prepared. The derivative **14** was also isolated from the Smith-degradation products of the ovalbumin glycopeptide after mercaptolysis.

INTRODUCTION

There are three possible monoaminotrioses, 1-amino-3-hydroxypropan-2-one[1], 3-amino-2-hydroxypropionaldehyde[2], and 2-amino-3-hydroxypropionaldehyde, and the last of these is the subject of this publication. This compound may exist transiently in solution during certain reactions[3,4], and its 2,4-dinitrophenylhydrazone sulphate was characterized amongst the degradation products of cycloserine[5]. The L-isomer of the *N*-benzoyl derivative is formed by oxidation of *N*-benzoyl-L-serine[6]. An attempt to isolate the *N*-acetyl derivative from an aqueous solution[7,8] gave a product that was probably polymeric. These preparations and an earlier one[9] all used periodate oxidation of amino sugar derivatives. Klenk and Faillard[10] isolated a compound from the degradation of acetylsphingosine, which was oxidised to *N*-acetylserine.

The formation of 2-amino-3-hydroxypropionaldehyde or its *N*-acetyl derivative from polysaccharides that contain a 2-acetamido-2-deoxyhexopyranoside residue having free hydroxyl groups in positions 3 and 4 can be expected after periodate oxidation, borohydride reduction, and acid hydrolysis. This reaction sequence will occur when the amino sugar is in a nonreducing, terminal position such as that of 2-acetamido-2-deoxy-D-glucose in ovalbumin[11], or 2-acetamido-2-deoxy-D-galactose

in the characteristic structure responsible for blood-group A specificity[12]. The amino sugar can become terminal after various degradations, as in orosomucoid[13]. The same fragment should occur after the degradation of mucin of the ovine submaxillary gland, where the 2-acetamido-2-deoxy-D-galactoside residue is attached by the 6-hydroxyl group to *N*-acetylneuraminic acid[14].

The C_3 and C_4 compounds having carbonyl, amino, and hydroxyl groups already described are only known in their ionic salt form (Table I). In this publication*, the preparation is described of derivatives of 2-amino-3-hydroxypropionaldehyde, together with the isolation of a derivative from the ovalbumin glycopeptide after Smith degradation.

TABLE I

HYDROCHLORIDES OF AMINO HYDROXY KETONES AND ALDEHYDES HAVING THREE AND FOUR CARBON ATOMS

Compound	*Properties*	*References*
$CH_2NH_2 \cdot CHOH \cdot CHO$, HCl	M.p. 143–145° (dec.) (a chloroplatinate is known)	2 and 16
$CH_2NH_2 \cdot CO \cdot CH_2OH$, HCl	M.p. 136–137°	1
2-Amino-2-deoxy-D-threose, HCl	Amorphous, approximately pure, furanose	17
2-Amino-2-deoxy-D-erythrose, HCl	M.p. 128°, furanose	17
2-Amino-3,3-dimethyl-4-hydroxybutyraldehyde, HCl	Crystalline, hygroscopic, furanose	18

RESULTS AND DISCUSSION

Reduction of ethyl 2-amino-3,3-diethoxypropionate[19] with lithium aluminium hydride gave the diethyl acetal **1** (95%) as a distillable syrup that was purified by recrystallization of its neutral oxalate and characterised as the bis(3,5-dinitrobenzoyl) derivative. Hydrolysis of compound **1** with acid is hindered by the quaternary ammonium group and requires prolonged treatment with concentrated hydrochloric acid.

In acid solution, an aldehyde such as $CH_2OH \cdot CH(\overset{+}{N}H_3) \cdot CHO$ is probably completely hydrated or polymerized. The n.m.r. spectrum in deuterium oxide does not show a signal corresponding to an aldehyde proton. The expected structures have four non-exchangeable protons, and two complex groups are distinguished in the spectrum, centred at 5.3 (one proton), and 4.0 p.p.m. (three protons). A very weak, residual signal in the methyl region enabled the extent of hydrolysis to be determined as 98%. The hydrolysis is confirmed by the reducing properties of the aqueous solution and by the preparation of various derivatives of the aldehyde function (see later). The unfavourable physical properties of the solid hydrochloride do not allow elemental analysis or determination of the infrared spectrum.

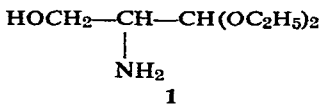
The preparation of several functional derivatives indicates that, at least under certain conditions, the compound reacts as the structure, $CH_2OH \cdot \overset{+}{C}HNH_3 \cdot CHO\ Cl^-$.

*Part of this work has already been communicated in preliminary notes[29–31].

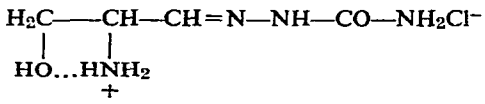
The semicarbazone hydrochloride was obtained by the usual method and has an analytical composition and ultraviolet and infrared spectra corresponding to the chelated structure **2**, which was confirmed by the n.m.r. spectrum[20]. The 2,4-dinitrophenylhydrazone hydrochloride (**3**) crystallizes with a molecule of methanol. The ethylene dithioacetal (**4**) was prepared by treatment with 1,2-ethanedithiol in concentrated hydrochloric acid and purified *via* its neutral oxalate. The structure of the dithioacetal was confirmed by reductive desulphuration to 2-aminopropan-1-ol[21]. The same product was also prepared from the diethyl acetal **1**.

TABLE II

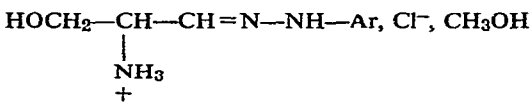
DERIVATIVES OF 2-AMINO-3-HYDROXYPROPIONALDEHYDE



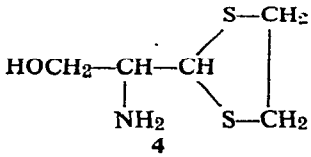
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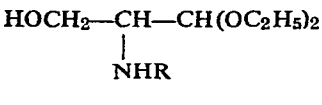
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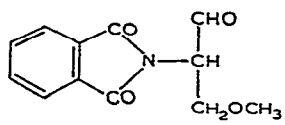
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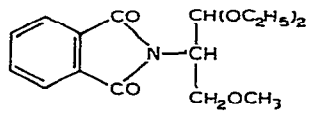
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5 R = $C_6H_5CH_2$
6 R = 2,4-dinitrophenyl

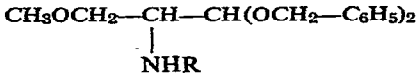


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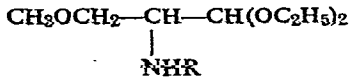


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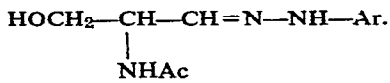
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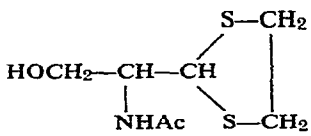
9 R = 2,4-dinitrophenyl



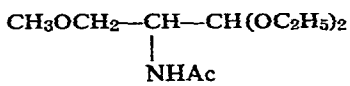
10 R = H
11 R = 2,4-dinitrophenyl
12 R = Ac



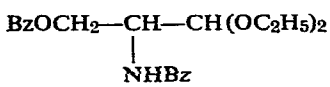
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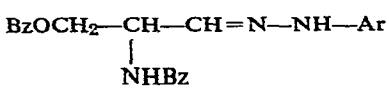
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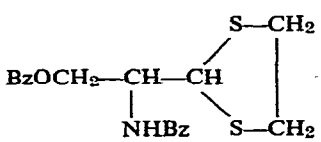
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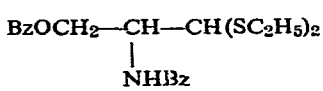
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19

With phenylhydrazine in dilute acetic acid, a low yield of hydroxypyruvaldehyde 1,2-bis(phenylhydrazone) was obtained (the yield in the case of glyceraldehyde is also low; 2-amino-2-deoxy-D-threose only gives 10% of D-*glycero*-tetrulose phenylosazone). The main product was an amorphous phenylhydrazone which on hydrogenation over Raney nickel yields 2,3-diaminopropan-1-ol, isolated as the picrate (yield, 20% from "2-amino-3-hydroxypropionaldehyde hydrochloride"). Traces of DL-serine were observed (paper chromatography) after oxidation with mercuric or silver oxide, and no glyceraldehyde was formed after treatment with nitrous acid.

Methyl and benzyl derivatives. — Ethyl 2-benzylamino-3,3-diethoxypropionate is also reduced by lithium aluminium hydride to yield the diethyl acetal **5**. Vigorous hydrolysis of **5** yielded an amorphous powder which is probably a hydrate or a polymer of $CH_2OH \cdot CH(\overset{+}{N}H_2 \cdot CH_2 \cdot C_6H_5) \cdot CHO\ Cl^-$. Treatment with phenylhydrazine, followed by reduction, gave 3-amino-2-benzylaminopropan-1-ol, isolated as the dipicrate.

Attempts to hydrolyse other acetal derivatives did not yield any α-aminoaldehyde products. The *N*-2,4-dinitrophenyl compound **6**, quantitatively obtained from acetal **1**, undergoes C–N rupture on acid hydrolysis to yield 2,4-dinitroaniline.

In order to gain access to the *O*-methyl series, 3-methoxy-2-phthalimidopropionaldehyde[23] (**7**) was converted into the dibenzyl acetal and treated with alcoholic hydrazine to yield 2-amino-1,1-dibenzyloxy-3-methoxypropane (characterized as the *N*-2,4-dinitrophenyl derivative **9**). Similarly, 1,1-diethoxy-3-methoxy-2-phthalimidopropane (**8**), with alcoholic hydrazine, yielded the unstable diethyl acetal **10** which gave no identified product on acid hydrolysis. Concentrated hydrochloric acid at room temperature degrades the corresponding *N*-2,4-dinitrophenyl acetal **11** to 2,4-dinitroaniline. At pH 2.5 and 90°, no hydrolysis was observed after one hour.

Acyl derivatives. — 2-Acetamido-2-deoxy-L-glyceraldehyde has already been prepared in aqueous solution[8], and in a powder form[7] (m.p. 92°), the analytical composition of which has not been determined. This powder was oxidised to serine after acid hydrolysis (yield not indicated), and it also yielded the 2,4-dinitrophenylosazone of pyruvaldehyde[7].

The acetamido acetal **12** is quantitatively obtained when a methanolic solution of the amino acetal **1** is treated by acetic anhydride. Compound **12** was hydrolysed by 0.1N sulphuric acid for five minutes at 100°, but the resulting aldehyde, $CH_2OH \cdot CH(NHCOCH_3) \cdot CHO$, could not be isolated. Paper chromatography indicated that the racemic compound in this solution has the same R_F value as the L-isomer[8]. Sodium borohydride reduction of the aldehyde in solution gives 2-acetamidopropane-1,3-diol[8] (overall yield of 75%). This is an indication of the high yield of the hydrolysis under the conditions chosen. Acetal **12** yielded a 2,4-dinitrophenylhydrazone **13** and an ethylene dithioacetal **14**.

The direct acetylation of the presumed compound, $CH_2OH \cdot \overset{+}{C}HNH_3 \cdot CHO\ Cl^-$, in methanol–acetic anhydride–triethylamine, yielded a white, amorphous solid, which did not give 2-acetamidopropane-1,3-diol with sodium borohydride, but afforded the 2,4-dinitrophenylosazone of pyruvaldehyde with the appropriate reagent[7].

The acetylation of acetal **10** gives the crystalline acetamido acetal **15** which can also be isolated, but in a lower yield, by treatment of the acetamido acetal **12** with methyl iodide and barium oxide in *N*,*N*-dimethylformamide. The aldehyde corresponding to the dibenzoyl derivative **16** cannot be obtained when the latter is hydrolysed. The 2,4-dinitrophenylhydrazone **17** is precipitated when the acetal **16** is left for a long time in the presence of the reagent dissolved in 2N hydrochloric acid. Attempts to hydrolyse the benzoyl dithioacetals **18** and **19** by mercuric chloride were unsuccessful.

1-Amino-3-hydroxypropan-2-one. — The preparation of 1-amino-3-hydroxypropan-2-one hydrochloride was repeated and confirmed[1]. The n.m.r. spectrum in deuterium oxide has two equal signals, at δ 4.18 and 4.49 p.p.m., which correspond to the two methylene groups. This spectrum is thus entirely different from that of "2-amino-3-hydroxypropionaldehyde hydrochloride". Moreover, the ketone gives a different semicarbazone, the structure of which is confirmed by its n.m.r. spectrum.

*Characterization of a derivative of 2-acetamido-2-deoxy-*D*-glyceraldehyde after degradation of the ovalbumin glycopeptide.* — The above results indicate that 2-acetamido-2-deoxy-D-glyceraldehyde is best characterised as the ethylene dithioacetal **14**. This derivative may be characterised chromatographically (see Experimental section) and by its mass spectrum. The parent peak is very weak (0.2% of the base peak), as observed in similar compounds[24]. The fragmentation in Fig. 1 is proposed by analogy with published results[25]. The protonated acetamide, $CH_3\text{-}C(^{+}OH)\text{=}NH_2$ (*m/e* 60), and the acetyl ion, $CH_3\text{-}CO^+$ (*m/e* 43), are also observed.

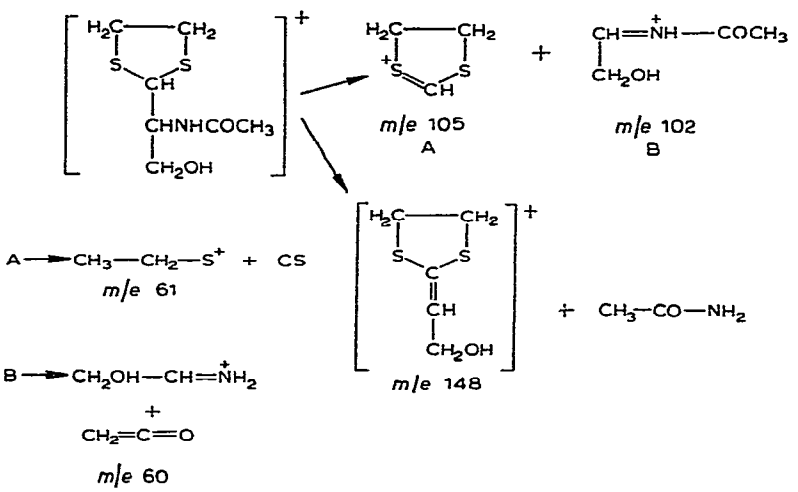


Fig. 1

The glycopeptide was prepared by pronase hydrolysis of ovalbumin, but without the final purification by successive treatments with benzyl chloroformate and carboxypeptidase[26]. It probably still contained traces of serine, threonine, and

especially leucine residues. Of the three 2-acetamido-2-deoxy-D-glucose residues present in this glycopeptide, only one is terminal and non-reducing, and thus open to attack by periodate[27]. The periodate oxidation and the borohydride reduction were carried out under the described conditions[27], but the mild, acid hydrolysis (final stage of the Smith degradation[15]) was replaced by a mercaptolysis with 1,2-ethanedithiol in concentrated hydrochloric acid at room temperature. This treatment does not break the *N*-acetyl bonds[25]. The reaction sequence is as follows:

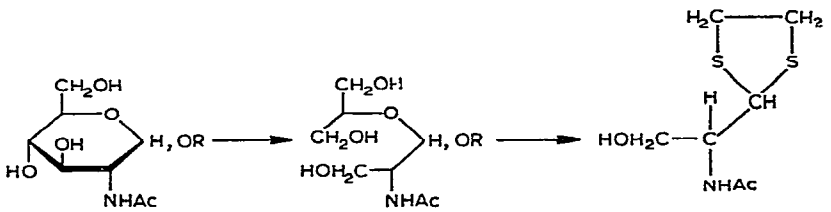


Fig. 2

These reactions were carried out on 410 mg of glycopeptide, from which 17 mg of compound **14** were obtained. This corresponds to a yield of *ca.* 30% if the molecular weight of the glycopeptide is taken as 1551.

EXPERIMENTAL

All solvents were removed under diminished pressure in a rotary evaporator at 45° unless otherwise stated. Melting points were determined on a Kofler hot-stage apparatus and are corrected. N.m.r. spectra were determined for solutions in D_2O at 60 MHz on a Varian A-60 spectrometer at 35°. Mass spectra were determined on an MS 9 instrument with a direct-insertion probe and operation at 70 ev. I.r. spectra were recorded with a Perkin–Elmer spectrophotometer (Model 237), either as thin films or Nujol mulls. U.v. spectra were measured with a Jobin and Yvon spectrophotometer (Model Maroc IV) at concentrations of about 10^{-4}M. Microanalyses were performed by the C.N.R.S. Laboratories, Thiais, France.

2-Amino-3-hydroxypropionaldehyde diethyl acetal (**1**). — A solution of ethyl 2-amino-3,3-diethoxypropionate (49 g) in dry ether (400 ml) was added with gentle stirring and mild reflux for 3 h to a suspension of lithium aluminium hydride (19.7 g) in dry ether (600 ml). Water (65 ml) was added to the reaction mixture cooled in an ice–salt bath. The ethereal layer was decanted off, and the inorganic residue was washed with boiling ethanol (5 × 200 ml). The ethanolic solution was evaporated to dryness at 35°, and the residue was taken up in a mixture of ethanol (350 ml) and dry ether (350 ml). This solution was stored for one night in a refrigerator and filtered. This filtrate, together with the former ethereal layer, was evaporated to dryness, and the brown, residual syrup was dried (P_2O_5, *in vacuo*) and distilled to give a colourless liquid (33.4 g, 85%) which solidified at 0°; b.p. 72°/0.2 mm, n_D^{25} 1.443.

Anal. Calc. for $C_7H_{17}NO_3$: C, 51.51; H, 10.50; N, 8.58. Found: C, 51.62; H, 10.51; N, 8.30.

The crude syrup (489 mg, 3 mmoles) was dissolved in ethanol (10 ml) and treated with oxalic acid dihydrate (189 mg, 1.5 mmoles) in ethanol (10 ml). The slight precipitate of lithium oxalate was filtered off, and anhydrous ether was added to the filtrate to give the neutral oxalate, m.p. 150–151° (from ethanol–ether).

Anal. Calc. for $C_{16}H_{36}N_2O_{10}$: C, 46.14; H, 8.71; N, 6.73. Found: C, 46.01; H, 8.65; N, 6.76.

An aqueous solution of the oxalate (5 g) was treated with saturated, aqueous barium hydroxide (75 ml). The filtered solution was evaporated, the residue was dissolved in ethanol, and the filtered solution was evaporated to give acetal **1** as a light-yellow syrup (3.74 g, 100%), b.p. 80–82°/0.5 mm.

The bis-*O,N*-(3,5-dinitrobenzoyl) derivative had m.p. 171–173° (dec.) (from ethanol–benzene).

Anal. Calc. for $C_{21}H_{21}N_5O_{13}$: C, 45.74; H, 3.84; N, 12.70. Found: C, 45.74; H, 3.77; N, 12.70.

With benzoyl chloride (4 equiv.) in 10% sodium hydroxide, acetal **1** gave a di-*O,N*-benzoyl derivative, m.p. 110° (from ethanol).

Anal. Calc. for $C_{21}H_{25}NO_5$: C, 67.90; H, 6.79; N, 3.77. Found: C, 67.76; H, 6.82; N, 3.66.

2-Amino-3-hydroxypropionaldehyde hydrochloride. — The acetal **1** (10 g) was added dropwise to conc. hydrochloric acid (20 ml) at −15°. The reaction mixture was kept for 15 h at room temperature, and then the hydrochloric acid was rapidly evaporated at ~15°/0.1 mm. The residual solid was treated with conc. hydrochloric acid (20 ml) at room temperature overnight, after which the hydrochloric acid was evaporated as above. The solid was washed several times with dry ether, finely ground, and dried *in vacuo* over KOH and P_2O_5 at 0°. The white, amorphous solid (quantitative yield) obtained was very hygroscopic, unstable in the atmosphere, soluble in water, slightly soluble in methanol, and insoluble in both ethanol and ether.

Semicarbazone hydrochloride **2**. — The above product (250 mg, 2 mmoles) was dissolved in water (5 ml) and treated with semicarbazide hydrochloride (223 mg, 2 mmoles) and sodium acetate trihydrate (272 mg, 2 mmoles). A small amount of biuret was filtered off, and the filtrate was heated for 1 h at 100°. The water was removed, and the residue was crystallised from methanol–ether; yield 75%; m.p. 155–156° (dec.); λ_{max}^{water} 228 nm (ε 8200); n.m.r.: ref. 20.

Anal. Calc. for $C_4H_{11}ClN_4O_2$: C, 26.31; H, 6.07; N, 30.68; HCl, 20.0. Found: C, 26.33; H, 6.32; N, 30.76; HCl, 20.1.

2,4-Dinitrophenylhydrazone hydrochloride **3**. — The amorphous aldehyde was dissolved in the minimal amount of methanol and treated with the reagent (1 equiv.) dissolved in methanol in the presence of a few drops of conc. hydrochloric acid; a light-red precipitate was discarded, and the filtrate was evaporated to dryness. The residue was dissolved in a little water, and the solution was filtered, and concentrated until yellow crystals appeared. These crystals were recrystallized with difficulty from methanol; yield, 47%; m.p. 187–189° (dec.).

Anal. Calc. for $C_9H_{12}ClN_5O_5 \cdot CH_3OH$: C, 35.53; H, 4.73; N, 20.73. Found: C, 34.75; H, 4.73; N, 20.91.

Phenylhydrazone and its reduction. — A solution of 2-amino-3-hydroxypropionaldehyde hydrochloride (900 mg) in water (9 ml) was treated with phenylhydrazine (775 mg) in 50% acetic acid (1 ml). A precipitate of hydroxypyruvaldehyde 1,2-bis-(phenylhydrazone) (m.p. 133–134°) was removed, and the filtrate was reduced by catalytic hydrogenation in the presence of Raney nickel at room temperature and atmospheric pressure. One mol of hydrogen was absorbed after 18 h, and the catalyst was then filtered off and washed with water. The aniline was extracted with benzene, the aqueous phase was evaporated, and the residue was treated with an aqueous solution of picric acid to yield 2,3-diaminopropan-1-ol dipicrate (20%), m.p. 216–217° (dec.); lit.[27], m.p. 215° (dec.).

Anal. Calc. for $C_{15}H_{16}N_8O_{15}$: C, 32.85; H, 2.94; N, 20.44. Found: C, 32.85; H, 3.37; N, 20.23.

Ethylene dithioacetal derivative **4**. — The amorphous aldehyde (1.2 g) dissolved in concentrated hydrochloric acid (10 ml) was treated at 0° with 1,2-ethanedithiol (1 g) and then stirred for 24 h at room temperature. The reaction mixture was poured on to a mixture of 40% aqueous sodium hydroxide (25 ml) and crushed ice (60 g). The product was extracted with chloroform, the extract was dried (Na_2SO_4) and evaporated, and distillation of the residue gave dithioacetal **4**; yield, 80%; b.p. 170–180°/0.2 mm; m.p. 60–63°.

An ethanolic solution of oxalic acid dihydrate (66 mg) was added to an ethanolic solution of the dithioacetal (173 mg) to give the neutral oxalate which crystallised after addition of a little dry ether; m.p. 175–178°.

Anal. Calc. for $C_{12}H_{24}N_2O_6S_4$: C, 34.27; H, 5.75; N, 6.66. Found: C, 34.31; H, 5.85; N, 6.57.

2-Acetamido-3-hydroxypropionaldehyde ethylene dithioacetal (**14**). — A solution of the above dithioacetal (1 g) in dry methanol (10 ml) was treated with acetic anhydride (1.4 ml) for 6 h at room temperature. The solution was evaporated, and the residue was triturated with toluene, which was then evaporated. The crystalline product (quantitative yield) had m.p. 137–138°; $\nu_{max}^{CHCl_3}$ 3430, 3280 (NH and OH); 3010; 2930; 1665 (amide I); 1510 (amide II); 1425; 1373; 1280; 1120; 1080; 1045 cm^{-1}. λ_{max}^{water} 234 nm (ε 320); and was soluble in chloroform. The mass spectrum exhibited the following principal peaks as a percentage of the base peak: *m/e* 148 (84); 134 (6); 120 (4); 107 (6); 106 (6); 105 (100); 102 (20); 92 (4); 87 (3); 84 (3); 74 (3); 61 (14); 60 (84); 59 (6); 58 (3); 45 (13); 43 (43); 42 (8); 31 (4); 30 (9). Parent peak at 207 (0.15).

Anal. Calc. for $C_7H_{13}NO_2S_2$: C, 40.58; H, 6.32; N, 6.76; S, 30.89. Found: C, 40.40; H, 6.24; N, 6.73; S, 30.62.

1-Amino-3-hydroxypropan-2-one semicarbazone hydrochloride. — A solution of 1-amino-3-hydroxypropan-2-one hydrochloride (250 mg, 2 mmoles) in methanol was treated with equivalent amounts of semicarbazide hydrochloride and sodium acetate trihydrate under reflux for 30 min. The solution was evaporated to dryness, and the

residue was crystallized from methanol to give the product, m.p. 161–162° (dec.). N.m.r. data: δ 4.83 (2-proton singlet) and δ 4.35 (2-proton singlet).

Anal. Calc. for $C_4H_{11}ClN_4O_2$: C, 26.31; H, 6.07; N, 30.68; Cl, 19.42. Found: C, 26.39; H, 6.11; N, 30.68; Cl, 19.37.

2-Benzylamino-3-hydroxypropionaldehyde diethylacetal (**5**). — A solution of ethyl 2-benzylamino-3,3-diethoxy-propionate[28] (20 g) in dry ether (200 ml) was added dropwise during 3 h to a stirred suspension of lithium aluminium hydride (5.6 g) in dry ether (200 ml). Water (11 ml) was added to the cooled (−15°) mixture which was then filtered. The residue was washed three times with boiling ethanol, and the combined organic filtrates were evaporated. Distillation of the residue (15.7 g) gave an initial fraction (0.7 g), b.p. 30–50°/0.2 mm, which was discarded, and then compound **5** as a light-yellow oil (10 g), b.p. 130–135°/0.2 mm, n_D^{25} 1.5020, which gave a picrate, m.p. 91–92° (from aqueous ethanol).

Anal. Calc. for $C_{20}H_{26}N_4O_{10}$: C, 49.79; H, 5.43; N, 11.61. Found: C, 49.72; H, 5.51; N, 11.85.

2-Benzylamino-3-hydroxypropionaldehyde hydrochloride, and reduction of its phenylhydrazone. — Acetal **5** (1.5 g) was added dropwise to conc. hydrochloric acid (6 ml) at −15°. The solution was kept for 4 h at room temperature, and then evaporated to give a syrup (1.2 g) which was dissolved in water (10 ml) and treated with phenylhydrazine (640 mg) dissolved in 50% aqueous acetic acid (0.25 ml). The hydroxypyruvaldehyde 1,2-bis(phenylhydrazone) (270 mg, m.p. 130–135°) was removed, and the filtrate was hydrogenated in the presence of Raney nickel at room temperature and atmospheric pressure. Hydrogen (1.1 equiv.) was absorbed after 18 h. The filtered solution was washed with benzene and then evaporated. The residue was dissolved in a little alcohol and treated with picric acid (1.53 g) dissolved in alcohol to give the dipicrate of 3-amino-2-benzylaminopropan-1-ol, m.p. 186–187° (dec.) (from ethanol–light petroleum).

Anal. Calc. for $C_{22}H_{22}N_8O_{15}$: C, 41.39; H, 3.47; N, 17.55. Found: C, 41.52; H, 3.62; N, 17.78.

2-(2,4-Dinitrophenylamino)-3-hydroxypropionaldehyde diethyl acetal (**6**). — The acetal **1** (1.63 g, 10 mmoles) was stirred for 24 h at room temperature with 1-fluoro-2,4-dinitrobenzene (1.86 g, 10 mmoles) and sodium carbonate decahydrate (1.43 g, 5 mmoles) in 50% aqueous acetone (80 ml). The mixture was evaporated, the residue was dissolved in ethanol, and, after removal of sodium fluoride, the filtrate was evaporated. The residue was recrystallized from ethanol–light petroleum to give acetal **6** as yellow needles (90%), m.p. 68–69°.

Anal. Calc. for $C_{13}H_{19}N_3O_7$: C, 47.41; H, 5.82; N, 12.76. Found: C, 47.60; H, 5.89; N, 12.82.

The hydrolysis of the above compound in a solution of acetone–5M hydrochloric acid (4:1) gave only 2,4-dinitroaniline, m.p. 179°, with a quantitative yield if the hydrolysis time was long enough.

2-(2,4-Dinitrophenylamino)-3-methoxypropionaldehyde dibenzyl acetal (**9**). — The phthalimido aldehyde[23] **7** (23.3 g) was heated under reflux in a Dean and Stark

apparatus with benzene (800 ml) in the presence of benzyl alcohol (25.2 g) and toluene-*p*-sulphonic acid (0.8 g). The cooled solution was washed with water, dried (Na_2SO_4), and evaporated. The syrupy residue (38 g) was dissolved in ethanol (350 ml) and treated under reflux for 3 h with an ethanolic solution (350 ml) of M hydrazine hydrate. On cooling, some of the phthalohydrazide precipitated, and the rest, after the addition of dichloromethane. The filtrate was evaporated to yield a yellow oil. This oil (600 mg) was characterized as 2-amino-3-methoxypropionaldehyde dibenzyl acetal by its reaction with 1-fluoro-2,4-dinitrobenzene (372 mg, 2 mmoles) and sodium carbonate (106 mg, 1 mmole) in 50% aqueous acetone (20 ml). The mixture was evaporated to dryness and the residue taken up several times in alcohol to yield bright-yellow crystals of acetal **9**, m.p. 85–86°.

Anal. Calc. for $C_{23}H_{23}N_3O_7$: C, 61.66; H, 5.39; N, 8.99. Found: C, 61.85; H, 5.54; N, 8.80.

3-Methoxy-2-phthalimidopropionaldehyde diethyl acetal (**8**). — The aldehyde **7** (11.5 g) was dissolved in the minimal amount of ethanol, and kept for a week in the presence of ethyl orthoformate (8 g) and anhydrous ammonium chloride (26 mg). The solution was evaporated, and the residue was taken up in water (100 ml) containing a few drops of conc. ammonia. The product was extracted by ether, and, after removal of the solvent, the residue was distilled to give the acetal **8** (12 g, 80%), b.p. 150°/0.1 mm, m.p. 44–45°.

Anal. Calc. for $C_{16}H_{21}NO_5$: C, 62.52; H, 6.88; N, 4.56. Found: C, 61.85; H, 6.80; N, 4.95.

2-Amino-3-methoxypropionaldehyde diethyl acetal (**10**). — An ethanolic solution (90 ml) of acetal **8** (12 g) was refluxed for 30 min with a M ethanolic solution (48.5 ml) of hydrazine hydrate. The cooled solution was filtered and evaporated, and distillation of the residue gave acetal **10** (5.6 g, 88%), b.p. 85°/12mm, which rapidly turned red in the atmosphere.

Anal. Calc. for $C_8H_{19}NO_3$: C, 54.21; H, 10.81; N, 7.90. Found: C, 54.21; H, 10.63; N, 8.40.

The 2-(2,4-dinitrophenyl) derivative **11** (74%) was prepared from acetal **10** by the method already described for **5**, to give golden-yellow needles, m.p. 72°.

Anal. Calc. for $C_{14}H_{21}N_3O_7$: C, 48.97; H, 6.17; N, 12.24; Found: C, 49.16; H, 6.10; N, 12.35.

2-Acetamido-3-hydroxypropionaldehyde diethyl acetal (**12**). — Acetic anhydride (0.9 ml) was added dropwise to a stirred solution of the acetal **1** (1 g) in dry methanol (10 ml). After 24 h at room temperature, the solution was evaporated, and the last traces of acetic acid were removed by addition of water and evaporation. The syrupy residue crystallised *in vacuo* over P_2O_5 at 0°. The crystals were washed with ice-cold light petroleum; m.p. 66–70°. The product decomposed on attempted distillation.

Anal. Calc. for $C_9H_{19}NO_4$: C, 52.66; H, 9.33; N, 6.82. Found: C, 51.83; H, 9.59; N, 7.16.

Preparation and reactions of 2-acetamido-3-hydroxypropionaldehyde. — (*a*) *Aqueous solution.* The acetal **12** (1.025 g) was heated for 5 min at 95° in 0.1N

sulphuric acid (25 ml), and the cooled solution was neutralized with barium carbonate and centrifuged.

(*b*) *Reduction.* The above supernatant was cooled to 0° and stirred with sodium borohydride (690 mg) dissolved in cold water (25 ml) for 30 min, and then for 2 h at room temperature. The solution was neutralized with dilute acetic acid and demineralized on Dowex-50 (H^+, 32 mequiv.) and Amberlite IR-45 (OH^-, 120 mequiv.) resin columns. The eluate was evaporated to dryness, and traces of boric acid were removed by repeated evaporation of methanol. The crystalline residue (0.5 g, 75%), m.p. 80–84°, was recrystallized from methanol–ethyl acetate (1:1) by adding light petroleum to give 2-acetamidopropane-1,3-diol, m.p. 87–88°; lit.[8], m.p. 89–80°.

Anal. Calc. for $C_5H_{11}NO_3$: C, 45.10; H, 8.32; N, 10.52. Found: C, 45.21; H, 8.27; N, 10.66.

(*c*) *2,4-Dinitrophenylhydrazone* **13**. The acetal **12** (205 mg, 1 mmole) in ethanol (2.5 ml) was added to a solution of the reagent (198 mg) in alcohol (50 ml) and 5% hydrochloric acid (15 ml). The reaction mixture was heated at 100° until the appearance of a precipitate, and then kept at 0° to yield yellow crystals, m.p. 196–201°.

Anal. Calc. for $C_{11}H_{13}N_5O_6$: C, 42.44; H, 4.21; N, 22.50. Found: C, 42.33; H, 4.22; N, 22.54.

2-Acetamido-3-methoxypropionaldehyde diethyl acetal (**15**). — (*a*) A mixture containing acetal **12** (1.23 g), methyl iodide (1.5 ml), and barium oxide (1.84 g) in *N,N*-dimethylformamide (20 ml) was stirred for 7 h at 20°. The product was extracted with chloroform (3 × 20 ml), and the chloroform solution was washed with water (3 × 10 ml) and evaporated. Distillation of the residue in a micro-distilling tube at 150°(bath)/0.1 mm gave acetal **15**, m.p. 49–54°.

Anal. Calc. for $C_{10}H_{21}NO_4$: C, 54.77; H, 9.65; N, 6.39. Found: C, 54.57; H, 9.36; N, 6.38.

(*b*) Treatment of the diethyl acetal **10** with acetic anhydride–methanol, as described for acetal **12**, gave acetal **15**, b.p. 89°/0.2 mm, m.p. 67°.

Anal. Calc. for $C_{10}H_{21}NO_4$: C, 54.77; H, 9.65; N, 6.39. Found: C, 54.70; H, 9.65; N, 6.47.

2-Benzamido-3-benzoyloxypropionaldehyde 2,4-dinitrophenylhydrazone (**17**). — Compound **16** (100 mg) was dissolved in alcohol and treated for a few minutes at 100° with the reagent (53 mg) dissolved in ethanol (10 ml) and 2N hydrochloric acid (3 ml) to give the title compound, m.p. 195° (from ethyl acetate).

Anal. Calc. for $C_{23}H_{19}N_5O_7$: C, 57.86; H, 4.01; N, 14.67. Found: C, 57.65; H, 4.18; N, 14.77.

2-Benzamido-3-benzoyloxypropionaldehyde ethylene dithioacetal (**18**). — A solution of the dithioacetal **4** (10 mmoles) in chloroform (25 ml) and pyridine (3.3 ml) was treated with benzoyl chloride (40 mmoles), in the usual way, to give compound **18**, m.p. 166°.

Anal. Calc. for $C_{19}H_{19}NO_3S_2$: C, 61.10; H, 5.13; N, 3.75. Found: C, 60.58; H, 5.11; N, 3.51.

2-Benzamido-3-benzoyloxypropionaldehyde diethyl dithioacetal (**19**). — The acetal **1** (3.26 g) was dissolved in ice-cold water (1.6 ml). To this solution was added, with vigorous stirring, conc. hydrochloric acid (14 ml), followed by ethanethiol (4.9 ml). After 24 h at room temperature, the reaction mixture was poured on to 40% sodium hydroxide (35 ml) and crushed ice (90 g). The product was extracted by chloroform, and the extract was dried (Na_2SO_4) and evaporated. A solution of the syrupy residue (3.72 g, 95%) in chloroform (50 ml) and pyridine (6.6 ml) was treated with benzoyl chloride (4 equiv.) by the usual method. The product was recrystallized from ethanol; yield, 5.1 g (63% total yield); m.p. 131–133°.

Anal. Calc. for $C_{21}H_{25}NO_3S_2$: C, 62.50; H, 6.24; N, 3.47. Found: C, 62.41; H, 6.22; N, 3.40.

Degradation of the ovalbumin glycopeptide. — The glycopeptide[26] (410 mg) was kept for 24 h at 5° in the dark in 0.05M sodium metaperiodate (50 ml). The excess of metaperiodate was then destroyed by treatment with ethylene glycol (1.4 ml) for 1 h at room temperature. Barium chloride dihydrate (611 mg) was then added, and the filtered solution was treated with sodium borohydride (4.5 g) for 24 h at 4–5°, brought to pH 7 with dilute hydrochloric acid, and evaporated at 30°. The residue (containing a great amount of sodium chloride and borate) was taken up in conc. hydrochloric acid (100 ml) and stirred for 48 h at room temperature with 1,2-ethanedithiol (5 ml). The hydrochloric acid and the excess of 1,2-ethanedithiol were rapidly evaporated at room temperature/0.1 mm. The residue was dried *in vacuo* over KOH and then taken up in dry methanol. The insoluble sodium salts were discarded, and the filtrate evaporated to dryness. A solution of the dry residue in chloroform was filtered and evaporated. The residue was chromatographically identical with racemic 2-acetamido-3-hydroxypropionaldehyde ethylene dithioacetal **14** in the following three systems: (*a*) paper chromatography (Whatman No. 1 paper) with butyl alcohol–acetic acid–water (5:2:3) and detection by iodine vapour; R_F 0.87. (*b*) t.l.c. (fluorescent silica gel) with isopropyl alcohol–water–ethyl acetate (24:12:65) and u.v. detection (254 nm); R_F 0.68; and benzene–ethanol (100:15); R_F 0.33.

The mercaptolysis product was purified by the last chromatographic system, using a 0.5-mm layer and eluting the band with boiling alcohol, to give a product (17 mg) having a mass spectrum that was identical with that of compound **14**. Although the racemic compound was only slightly soluble in chloroform, the i.r. spectra of the two compounds in chloroform were identical except that the NH and OH bands (3430 and 3280 cm^{-1}) were not resolved for the D-isomer.

2-Acetamido-2-deoxy-D-glucose ethylene dithioacetal. — 1,2-Ethanedithiol (1.9 ml, 22.7 mmoles) was added to a stirred solution of 2-acetamido-2-deoxy-D-glucose (4.42 g, 20 mmoles) in conc. hydrochloric acid (20 ml) at 0°. The stirring was continued for 20 h at room temperature. The solution was diluted with methanol (200 ml), neutralized with lead carbonate, filtered, concentrated, and again filtered. Light petroleum was added until turbidity, and the product was recrystallized from ethyl acetate–methanol (1:1) to give the title compound, m.p. 168–169°, which was soluble in methanol and hardly soluble in ethyl acetate and chloroform. The product

had R_F (t.l.c.) 0.37 in isopropyl alcohol–water–ethyl acetate (24:12:65) and 0.04 in benzene–ethanol (100:15).

Anal. Calc. for $C_{10}H_{19}NO_5S_2$: C, 40.38; H, 6.44; N, 4.71; S, 21.56. Found: C, 40.67; H, 6.33; N, 4.57; S, 21.91.

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THE BIOSYNTHESIS OF STARCH

PART XIII*. INHIBITION OF POTATO PHOSPHORYLASE BY DONOR SUBSTRATE ANALOGUES**

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ABSTRACT

The inhibitory effect of D-glucose, 2-deoxy-D-*arabino*-hexose, 3-*O*-methyl-D-glucose, and 6-*O*-methyl-D-glucose on the enzymic synthesis catalysed by potato phosphorylase has been investigated. Kinetic methods showed that, for both of the substrates employed, a competitive inhibitor effect was exerted by each of the substrate analogues examined.

Values for K_i and K_m calculated from the experimental data indicate that, in the presence of each of the added substrate analogues, the affinity of the enzyme for both substrates decreased. On the basis of the changes in K_i values, it appeared that, in the case of each substrate, the inhibitor effect of D-glucose was suppressed by the absence of the hydroxyl groups at the positions 2 and 3.

INTRODUCTION

In earlier work on the mechanism of the action of potato phosphorylase, kinetic methods were used to determine the functional groups responsible for the binding of the substrate[1,2]. These groups were identified by inhibition tests[2,3]. Later, in order to investigate the nature of the interacting groups in the substrate, various derivatives of D-glucose and D-glucopyranosyl phosphate were prepared[4], and their effect on the enzyme reaction was studied[5]. This paper reports the inhibitory effects of donor substrate analogues.

Previous papers in this field discuss only the effect of D-glucose, but the results are contradictory. According to Green and Stumpf[6], Summer[7], Ran[8], and Nakamura[9], the enzymic reaction is not inhibited by D-glucose, whereas Fischer and Hilpert[10], Arreguin[11], and Porter[12] reported that D-glucose causes a competitive inhibition. Thus, prior to investigating the functional groups of the substrates, it appeared necessary to establish the nature of the inhibitory effect of D-glucose[1]. After these preliminary investigations, we examined the role of the hydroxyl groups at positions 2, 3, and 6, on the basis of the inhibition data obtained with suitable derivatives of D-glucose.

*Part XII: Stärke, in the press.
**71st Communication on polysaccharide research from this Department.

EXPERIMENTAL AND RESULTS

Materials. — *Potato phosphorylase* was prefractionated with ammonium sulphate and purified by chromatography on DEAE-cellulose[13] and had a specific activity of 450 E units per mg of protein. *Amylopectin* was an analytical grade (Calbiochem) product, and the D-*glucopyranosyl phosphate* (potassium salt, analytical grade) was a product of the Reanal Factory of Fine Chemicals, Budapest. The following substrate analogues were used: D-glucose (analytical grade, Merck), 2-deoxy-D-*arabino*-hexose (analytical grade, Reanal Factory of Fine Chemicals), 3-*O*-methyl-α-D-glucose[14], and 6-*O*-methyl-D-glucose (obtained from the Institute of Organic Chemistry of L. Kossuth University, Debrecen).

The substrates and the substrate analogues were employed in the inhibition experiments as 0.1M solutions in a citrate buffer of pH 6.0.

Determination of the initial velocity (v_0). — The inhibition mixture, which contained the enzyme, the donor and acceptor substrates, and, in certain experiments, the corresponding substrate analogue, was placed, at the end of the reaction period, for 10 min in a water bath at 100°. After inactivation of the enzyme and removal of the denaturated protein by centrifugation, the amount of liberated inorganic phosphate was determined by the Fiske–Subbarow method[16] as modified by Butenko and Kirsch[15]. From the quantity of liberated inorganic phosphate (first-order reaction) and the value of the equilibrium constant[17], the initial velocity can be calculated.

Determination of the mechanism of inhibition and of the inhibition constant. — For the determination of the mechanism of inhibition, the graphical method suggested by Lineweaver and Burk[18] was employed. Their equation for the inhibited reaction can be rearranged as follows:

$$\frac{1}{v_i} = \frac{1}{V_m} + \frac{K_m}{V_m[S]}\left[1 + \frac{[I]}{K_i}\right],$$

where v_i is the initial velocity of reaction, V_m the maximum velocity of reaction, K_m the apparent Michaelis constant, $[S]$ the substrate concentration, K_i the inhibition constant, and $[I]$ the inhibitor concentration.

According to the above equation, the resulting straight lines intersect the ordinate at $1/V_m$, and the abscissa at $1/K_m$. The dissociation constant of the enzyme–inhibitor complex, and the inhibition constant K_i can be calculated, in turn, from the slope of the graph.

The linear equation for a non-inhibited system can be written in a completely analogous way. The values V_m and K_m can be determined in a manner analogous to the previous procedure.

Technique of inhibition experiments. — In this series of experiments, the enzymic reaction was inhibited by D-glucose and by D-glucose analogues substituted at the 2-, 3-, and 6-positions.

In the course of the kinetic investigations, the concentrations of both D-glucopyranosyl phosphate (as donor substrate) and of amylopectin (as acceptor substrate) were varied, on applying two different, but constant, inhibitor concentrations.

Initial velocities of reaction have been determined, in both cases, in non-inhibited reaction systems (under identical conditions). The inhibiting effect of the substrate analogues was investigated only in the course of the enzymic synthesis catalysed by potato phosphorylase. It was established in the preliminary experiments that the phosphorolytic reaction is not inhibited by D-glucose and the above-mentioned substrate analogues[19].

Investigation of the inhibition by D-*glucose.* — In the synthesis reaction, the amount of inorganic phosphate liberated was determined at four different concentrations (3, 4, 10, and 20 mmoles per litre) of D-glucopyranosyl phosphate, at four different concentrations (0.095, 0.142, 0.237, and 0.950 mmole per litre) of non-reducing end groups of amylopectin and at two D-glucose concentrations (0.3 and 0.6 mole per litre).

The applied amount of enzyme was always 7.4 enzymic units, whilst the volume of reaction mixture totalled 2.0 ml. The following digest conditions were employed: 0.1M citrate buffer (pH 6.0) at 35 $\pm$0.1° for 10 min.

The v_0 values calculated from the measured data are listed in Table I.

Values for $1/v_0$ in the inhibited and non-inhibited reaction mixtures plotted against donor and acceptor substrates, respectively, are presented in Fig. 1.

TABLE I

INHIBITING EFFECT OF D-GLUCOSE ON INITIAL VELOCITIES AT VARIOUS SUBSTRATE CONCENTRATIONS

Substrate concentration (mmoles/litre)		$v_0 \cdot 10^{-3}$ (*μmoles/min*)		
D-*Glucopyranosyl phosphate*	*Amylopectin*[a]	*Without any inhibitor*	*With* D-*glucose* (*0.3 mole*)	(*0.6 mole*)
20	0.095	100.0	77.0	56.0
20	0.142	118.0	100.0	80.5
20	0.237	133.0	118.0	103.0
20	0.95	193.0	167.0	140.0
10	0.95	164.0	154.0	94.5
5	0.95	156.0	109.0	69.7
3	0.95	130.0	80.0	44.3

[a]Mmole of non-reducing end-groups/litre.

*Investigation of the inhibition by 2-deoxy-*D-arabino-*hexose.* — In this series of synthesis reactions, 0.3 and 0.6 mole of 2-deoxy-D-*arabino*-hexose were used as inhibitor. The v_0 values calculated from the data of measurements are presented in Table II. The reciprocal values of v_0 plotted against the reciprocal values of substrate concentrations are shown in Fig. 2.

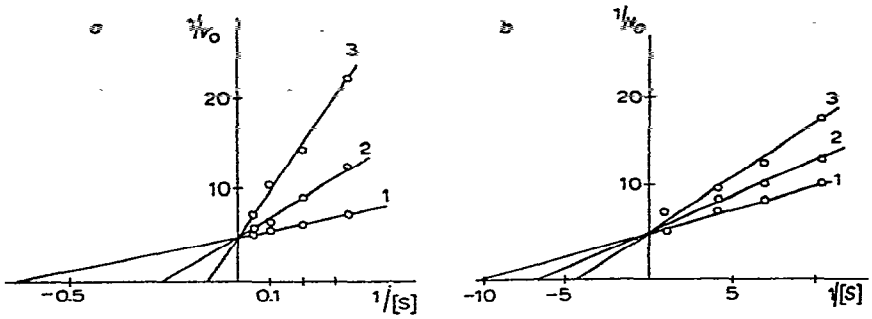


Fig. 1. Initial rate plotted against donor and acceptor substrate concentrations in the presence of D-glucose inhibitor. 1, No inhibitor; 2, with 0.3M D-glucose; 3, with 0.6M D-glucose; a, D-glucopyranosyl phosphate concentration varying from 3 to 20 mmoles/litre at a constant amylopectin concentration of 0.95 mmole of non-reducing end-group/litre; b, amylopectin concentration varying from 0.095 to 0.95 mmole of non-reducing end-group/litre at a constant D-glucopyranosyl phosphate concentration of 20 mmoles/litre.

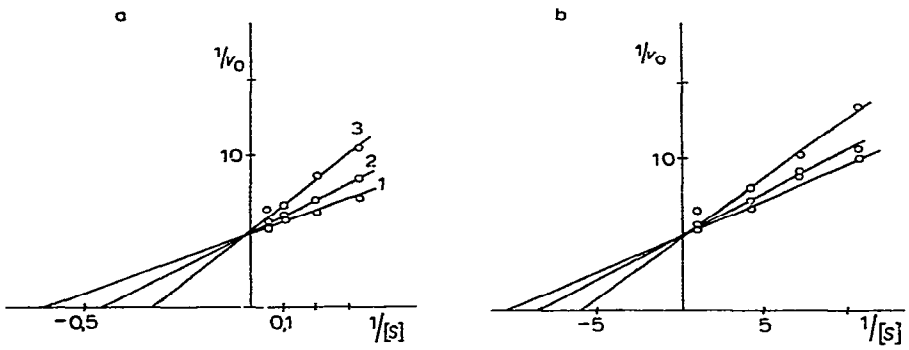


Fig. 2. Initial rate plotted against donor and acceptor substrate concentration in the presence of 2-deoxy-D-*arabino*-hexose inhibitor. 1, No inhibitor; 2, with 0.3M 2-deoxy-D-*arabino*-hexose; 3, with 0.6M 2-deoxy-D-*arabino*-hexose; a and b, see legend for Fig. 1.

TABLE II

INHIBITING EFFECT OF 2-DEOXY-D-*arabino*-HEXOSE ON INITIAL VELOCITIES AT VARIOUS SUBSTRATE CONCENTRATIONS

Substrate concentration (mmoles/litre)		$v_0 \cdot 10^{-3}$ (*μmoles/min*)		
D-*Glucopyranosyl phosphate*	*Amylopectin*[a]	*Without any inhibitor*	*With 2-Deoxy*-D-arabino-*hexose* (*0.3 mole*)	(*0.6 mole*)
20	0.095	100.0	95.5	73.2
20	0.142	109.0	109.0	88.5
20	0.237	146.5	136.0	123.0
20	0.95	181.0	176.5	148.0
10	0.95	166.0	162.0	146.0
5	0.95	156.0	141.5	113.0
3	0.95	138.0	116.5	95.0

[a]Mmole of non-reducing end-groups/litre.

Investigation of the inhibition by 3-O-methyl-D-glucose. — Because of the small amount of 3-*O*-methyl-D-glucose available, the inhibition was investigated only at one concentration (0.6M) of substrate analogue. The v_0 values determined from the results obtained are given in Table III.

Values of $1/v_0$ plotted against the two substrate concentrations are shown in Fig. 3.

TABLE III

INHIBITING EFFECT OF 3-*O*-METHYL-D-GLUCOSE ON INITIAL VELOCITIES AT VARIOUS SUBSTRATE CONCENTRATIONS

Substrate concentration (mmoles/litre)		$v_0 \cdot 10^{-3}$ (*μmoles/min*)	
D-Glucopyranosyl phosphate	*Amylopectin*[a]	*Without any inhibitor*	*With 0.6 mole of 3-O-methyl-D-glucose*
20	0.095	105.0	88.5
20	0.142	117.0	73.0
20	0.237	125.0	92.5
20	0.95	153.0	132.0
10	0.95	138.0	115.0
5	0.95	136.0	118.0
3	0.95	102.0	77.5

[a]Mmole of non-reducing end-groups per litre.

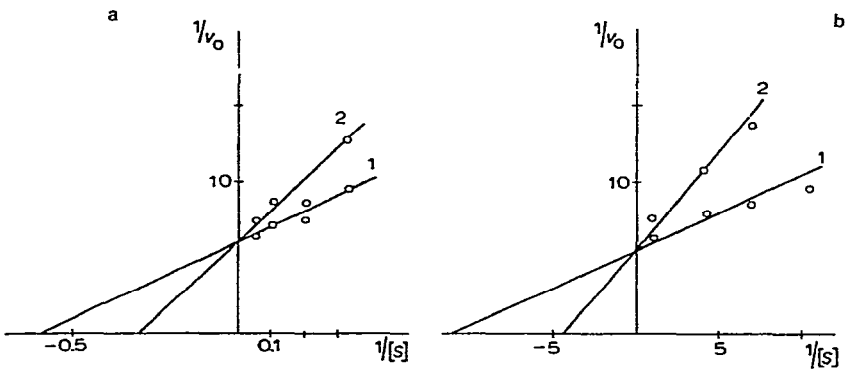


Fig. 3. Initial rate plotted against donor and acceptor substrate concentration in the presence of 3-*O*-methyl-D-glucose inhibitor. 1, No inhibitor; 2, with 0.6M 3-*O*-methyl-D-glucose; a and b, see legend for Fig. 1.

Investigation of the inhibition by 6-O-methyl-D-glucose. — Again, the effect of inhibition has been investigated only at one concentration (0.3M) of substrate analogue. On varying the donor substrate concentration (20, 10, 5, and 3 mmoles per litre), the acceptor substrate concentration was maintained constant (0.095 mmole per litre). On varying the concentration of amylopectin (0.095, 0.142, 0.237, and 0.95 mmole of non-reducing end-groups per litre), the concentration of D-glucopyranosyl phosphate was kept constant (20 mmoles per litre).

The calculated values of v_0 are presented in Table IV, and the values of $1/v_0$ plotted against substrate concentrations are shown in Fig. 4.

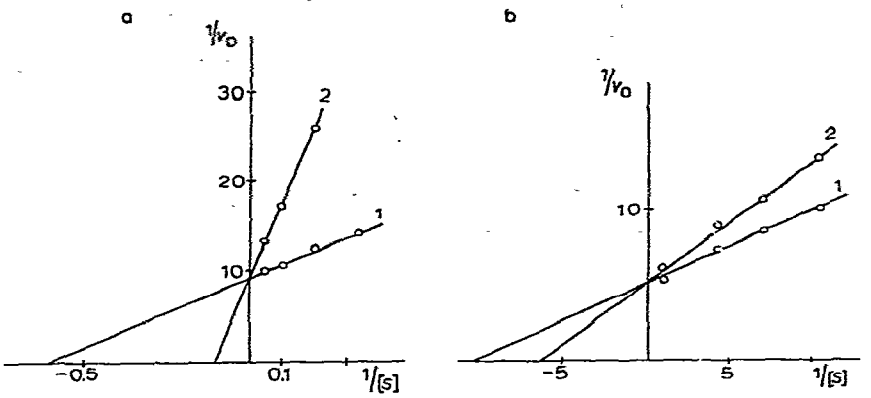


Fig. 4. Initial rate plotted against donor and acceptor substrate concentration in the presence of 6-*O*-methyl-D-glucose inhibitor. 1, No inhibitor; 2, with 0.3M 6-*O*-methyl-D-glucose; a and b, see legend for Fig. 1.

TABLE IV

INHIBITING EFFECT OF 6-*O*-METHYL-D-GLUCOSE ON INITIAL VELOCITIES AT VARIOUS SUBSTRATE CONCENTRATIONS

Substrate concentration (mmoles/litre)		$v_0 \cdot 10^{-3}$ (*μmoles/min*)	
D-*Glucopyranosyl phosphate*	*Amylopectin*[a]	*Without any inhibitor*	*With 0.3 mole of 6-O-methyl-D-glucose*
20	0.095	100.0	74.0
20	0.142	118.0	91.5
20	0.237	133.0	111.0
20	0.95	193.0	160.0
10	0.095	92.0	57.3
5	0.095	79.5	38.5
3	0.095	71.0	—

[a]Mmole of non-reducing end-groups per litre.

DISCUSSION

Evaluation of the results of investigations of inhibition. — As mentioned above, the results obtained were plotted by the double, reciprocal, graphic method suggested by Lineweaver and Burk (Figs. 1–4), and the values K_I, K_m, and V_m were established from the slope tangents of the plotted straight lines and from the horizontal and vertical axes (Table V). From these results, the conclusion is drawn that the inhibition process is competitive in respect of both the applied donor and the acceptor substrates for each of the substrate analogues employed. This is proved also by the fact that the values for V_m were identical throughout the experiments.

TABLE V

VALUES FOR K_m, K_i, AND V_m CALCULATED BY THE GRAPHIC METHOD OF LINEWEAVER AND BURK[18] FOR VARIOUS SUBSTRATE ANALOGUES

Inhibitor applied	*Inhibitor concentration (mole/litre)*	D-*Glucopyranosyl phosphate*			*Amylopectin*[a]		
		K_m *(mmoles per/litre)*	K_i	V_m *(μmole per sec)*	K_m *(mmole per litre)*	K_i	V_m *(μmole per sec)*
D-Glucose	0.0	1.64	—	0.204	0.220	—	0.204
	0.3	4.53	0.170	—	0.330	0.540	—
	0.6	10.00	0.115	—	0.500	0.405	—
2-Deoxy-D-*arabino*-D-hexose	0.0	1.60	—	0.200	0.097	—	0.202
	0.3	2.15	0.770	—	0.117	1.30	—
	0.6	3.33	0.520	—	0.168	0.79	—
3-*O*-Methyl-D-glucose	0.0	1.67	—	0.157	0.093	—	0.180
	0.3	—	—	—	—		—
	0.6	3.20	0.650	—	0.213	1.57	—
6-*O*-Methyl-D-glucose	0.0	1.65	—	0.167	0.094	—	0.190
	0.3	9.10	0.130	—	0.145	0.52	—

[a] Mmole and μmole of non-reducing end-groups.

The apparent Michaelis constants increased with the rise of inhibitor concentration both for the donor substrate and for the acceptor substrate. This means that, in the presence of an inhibitor, the affinity of the enzyme decreases for both types of substrate applied.

It appears from the data in Table V that, in the case of both substrates, the inhibitor effect of D-glucose is suppressed to a great extent by the absence of the hydroxyl groups at C-2 and C-3, though, in the latter case, it is likely that the steric hindrance of the methoxyl group also plays a role.

The absence of a hydroxyl group at C-6, in turn, caused no alterations in the inhibitor effect of D-glucose with any of the substrates examined.

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THE ACIDIC MUCOPOLYSACCHARIDES OF HUMAN SKIN

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ABSTRACT

The problems of extraction of acidic mucopolysaccharides from fibrous, human skin have been overcome, and a procedure has been established. The mucopolysaccharides so obtained have been fractionated by anion exchange. Determination of the hexuronic acid, 2-amino-2-deoxyhexose, and sulphate contents, and identification of the constituent 2-amino-2-deoxyhexoses of these polysaccharides have established the presence of three fractions containing hyaluronic acid, and of chondroitin 4-sulphate, chondroitin 6-sulphate, and dermatan sulphate in human skin. The characterisation of these polysaccharides on a basis of chemical structure has been made on 150-μg samples by using micro methods.

INTRODUCTION

With the advent of a method capable of fractionating acidic mucopolysaccharides[1] and with the micro methods evolved for their structural identification[2], it is now possible to overcome difficulties imposed by the limited availability of fresh tissue. A method for extraction and characterisation of the acidic mucopolysaccharides of human skin is now reported.

METHODS AND RESULTS

Direct application to human skin of the method designed for extraction of acidic mucopolysaccharides from non-fibrous tissue[1] did not extract a mucopolysaccharide. Attempts to break down the bonding of the fibrous material of human skin with such agents as acetic acid, 2-mercaptoethanol, and hydroxylamine, followed by proteolysis with pronase and action of alkali to liberate attached carbohydrate, did not improve the yield of mucopolysaccharide. However, the important factor in obtaining the best yield of carbohydrate was the initial state of fine division of the tissue. The necessity for an alkali-extraction stage was evident, since, in a tissue culture experiment with sodium sulphate-^{35}S, treatment of the dermis and epidermis with alkali extracted 95 and 25%, respectively, of the total radioactivity obtained in the polysaccharide fraction from these two layers of human skin.

The final experimental procedure was a much modified version of the original[1,3]. Fresh, human skin, prehomogenised with the aid of a Biox X-press (AB Biox, Nacka, Sweden), was defatted by solvent extraction. The tissue was then suspended in the enzyme-digestion buffer and ground in a series of semi-automated, glass tissue-grinders. The homogenate was incubated at 37° under sterile conditions with pronase-P (Cambrian Chemicals Ltd, London) for 5 days, with a second addition of pronase after 2 days. The residue from the centrifuged incubate was treated with 0.5N sodium hydroxide for 30 min at 37°. The solution/suspension was immediately cooled, neutralised with acetic acid, and centrifuged. The two supernatants were combined, and the extraction procedure was completed by denaturation of residual protein, dialysis of the supernatant, and concentration by rotary evaporation *in vacuo* at 30° (bath temperature).

Fractionation of the mucopolysaccharides. — The concentrated polysaccharide extract was fractionated[1] on De-Acidite FF resin (7–9% cross-linked, 100–200 mesh, Cl^-), and the dialysed fractions were scanned for uronic acids (carbazole[4]), protein (ninhydrin[5]), and u.v. absorption at 280 nm (Fig. 1). The fractions were also scanned for pentose[6], sialic acids[7], and uronic acid/hexose[8]; these analyses gave, respectively, weakly positive peaks corresponding to each uronic acid peak, negative results throughout, and no additional peaks. The fractions associated with carbazole-positive peaks were individually combined, and concentrated by rotary evaporation *in vacuo* at 30°.

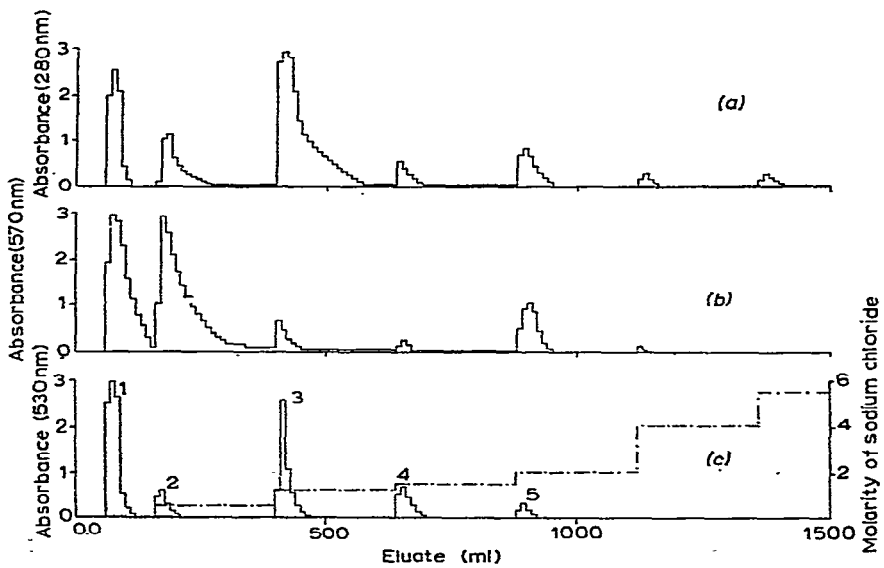


Fig. 1. Fractionation of human-skin polysaccharides on De-Acidite FF. (———), (*a*) u.v.-assay; (*b*) ninhydrin assay; (*c*) carbazole assay; (—.—.—), gradient.

The products from peaks 1 and 2 were further purified by fractionation on a column of DEAE-Sephadex A-50 (40–120 μ, Cl^-). Fractions were scanned for uronic

acid, protein, and u.v.-active material (Figs. 2 and 3). Separate elution of authentic samples of chondroitin and heparan sulphate from the De-Acidite FF column, under similar conditions, revealed that their elution positions coincided with peaks 2 and 3, respectively (see, however, chemical characterisation of these peaks).

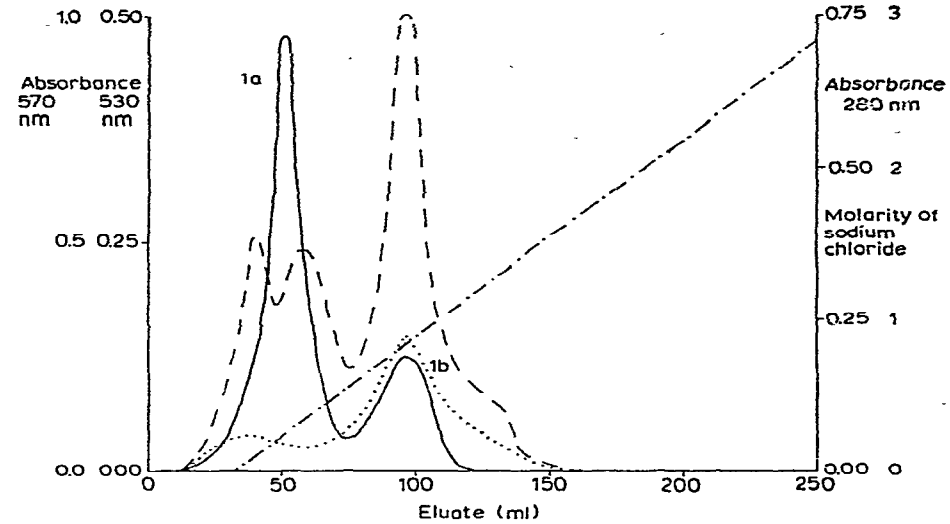


Fig. 2. Fractionation of polysaccharide 1 on DEAE-Sephadex. (———), carbazole assay; (— — —) ninhydrin assay; (.....), u.v. assay; (—·—·—), gradient.

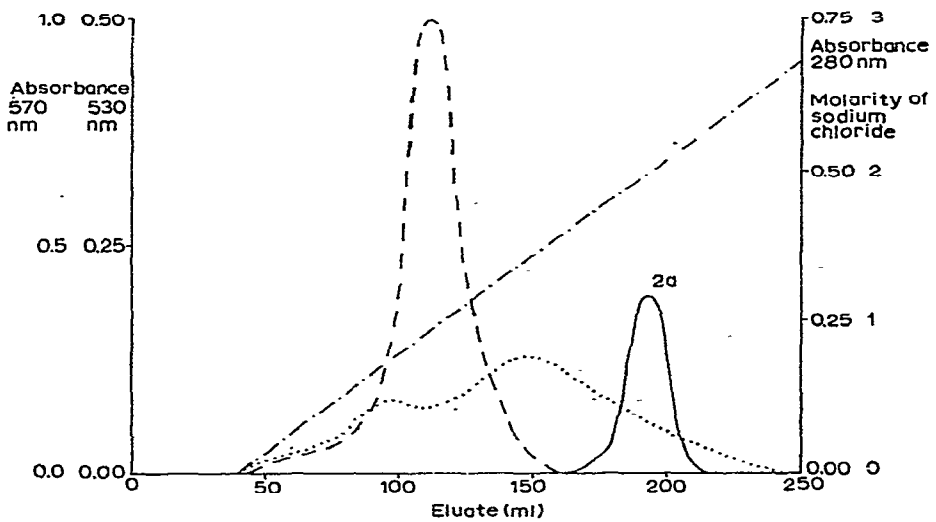


Fig. 3. Fractionation of polysaccharide 2 on DEAE-Sephadex. (———), carbazole assay; (— — —), ninhydrin assay; (.....), u.v. assay; (—·—·—), gradient.

Characterisation of the mucopolysaccharides. — The major peaks obtained from the fractionation were characterised by the established procedures[2].

(*a*) *Identification of 2-amino-2-deoxyhexose.* The polysaccharides were hydrolysed in 3.9N hydrochloric acid, and the liberated 2-amino-2-deoxyhexoses were characterised by chromatography on BioRad AG-50W x8 resin (samples **1a**, **1b**, and **2a**, 2-amino-2-deoxyglucose; samples **3** and **4**, 2-amino-2-deoxygalactose). Oxidation with ninhydrin gave the corresponding pentoses, which were identified by t.l.c. (butanone–acetic acid–saturated, aqueous boric acid, 9:1:1 by volume), and confirmed these assignments (samples **1a**, **1b**, and **2a**, arabinose; samples **3** and **4**, lyxose).

(*b*) *Uronic acid:2-amino-2-deoxyhexose:sulphate ratios.* The polysaccharides were hydrolysed in 3.9N hydrochloric acid at 100° for various times, and the liberated 2-amino-2-deoxyhexoses were extracted by the Boas[9] method on a diminished scale. The Elson–Morgan[10,11] assay showed that maximal liberation occurred after 9 h. Uronic acid was determined by the carbazole assay and sulphate by the flame photometric method[12]. The results from each assay are expressed in terms of molar ratios (Table I).

TABLE I

MOLAR RATIOS OF CONSTITUENTS

Sample	*Uronic acid*	*2-Amino-2-deoxyhexose*	*Sulphate*
1a	1.09	1.00	0.00
1b	0.33	1.00	2.31
2a	0.91	1.00	0.00
3	1.05	1.00	1.28
4	0.89	1.00	1.05
5	0.71	1.00	1.12

(*c*) *Susceptibility of the polysaccharides to hyaluronidase.* The polysaccharide solutions were treated with testicular hyaluronidase (Sigma Chemical Co., St. Louis, Missouri), and the incubates were analysed for the release of reducing 2-acetamido-2-deoxyhexoses by the Morgan–Elson[13,14] assay. Polysaccharides **1a**, **1b**, **2a**, and **4** gave positive results, whereas polysaccharides **3** and **5** gave negative results. Further aliquots of the incubates for polysaccharides **3** and **5** were fractionated on the calibrated[2] BioGel P-30 column. Uronic acid assay of the eluates corresponding to void volume, polymer, and oligomers demonstrated that polysaccharide **3** had been completely hydrolysed to oligomers, and that polysaccharide **5** remained intact.

(*d*) *Susceptibility of the polysaccharides to chondroitinase and sulphatases.* Chondroitinase powder was added to solutions of polysaccharides **3**, **4**, and **5** in 0.025M Tris buffer (pH 8.0), and, after incubation for 24 h at 37°, aliquots were assayed by the Warren[7] technique for the release of 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose (*A*). Each sample gave a positive result, indicating the formation of β-formylpyruvic acid at the periodate-oxidation stage of the assay. Fractionation on the calibrated BioGel P-30 column demonstrated that each sample had been degraded by the chondroitinase

and that no polymer remained. Fractionation of further aliquots on a BioRad AG-1 x8 resin micro-column, calibrated[2] for the elution position of disaccharide *A*, gave, in each case, one peak corresponding to this disaccharide, when the fractions were assayed by the carbazole method. The fractions associated with these peaks were individually combined and concentrated *in vacuo* to remove ammonium acetate. The products of hydrolysis with N sulphuric acid for 0–3 h at 100° were determined by the Boas–Elson–Morgan[9] assay. The absorption maxima (λ_{max}) for the chromophores of the samples and a 2-amino-2-deoxy-D-galactose standard were determined (Table II).

TABLE II

HYDROLYSIS OF THE DERIVED DISACCHARIDES

Sample		*Hydrolysis time* (h)				
		0.0	*0.5*	*1.0*	*2.0*	*3.0*
2-Amino-2-deoxy-D-glucose	Absorbance at 540 nm	0.31	0.31	0.31	0.31	0.30
	λ_{max} (nm)	540	540	540	540	540
3	Absorbance at 530 nm	0.15	0.37	0.43	0.43	0.49
	λ_{max} (nm)	525	527	530	530	538
4	Absorbance at 490 nm	0.10	0.31	0.38	0.38	0.42
	λ_{max} (nm)	490	490	490	490	520
5	Absorbance at 525 nm	0.04	0.14	0.19	0.19	0.23
	λ_{max} (nm)	525	525	525	525	530

DISCUSSION

The earlier procedure[1] for the extraction of mucopolysaccharides was designed for the skin of new-born rats, which is relatively easily homogenised and is solubilised by proteolytic hydrolysis. The failure of this method when applied to the skin of adult humans was attributed to the presence of fibrous collagen bundles to which the acidic mucopolysaccharides are covalently bound. The use of alkali is often necessary to free the carbohydrate from attached protein or residual peptides[15], since alkali will break the pentose–amino acid bond of the carbohydrate–protein linkage[16]. Hence, the original method was modified to include de-bonding agents and extraction with alkali. Since, at some stage, each of these extraction procedures gave a gelatinous mass with subsequent loss of polysaccharide along with removal of protein, it was evident that the collagen was still resisting the extraction. For extensively homogenised tissue, the extraction procedure, including the use of pronase and alkali, did not result in coagulation of the tissue fragments, and subsequent fractionation revealed that the acidic mucopolysaccharides had been isolated. A fine homogenate of the tissue, allowing full attack by the carbohydrate–protein splitting agents, was therefore necessary to give the maximal yield of polysaccharide.

The small content of pentose in the uronic acid-positive peaks revealed by

ion-exchange fractionation of the final extract could have arisen from nucleic acid, from the xylose/arabinose originally involved in the carbohydrate–protein link, or by decarboxylation of uronic acid. The presence of sialic acids in some mucopolysaccharide preparations has been reported[17], but, if these carbohydrates had been present, their removal by the enzyme would have been expected, since pronase contains a sialase[18].

When fractionated on DEAE-Sephadex, polysaccharides **1** and **2** both gave a carbohydrate peak (**1a** and **2a**) which contained equimolar amounts of 2-amino-2-deoxyhexose and hexuronic acid, but no sulphate. These results are consistent with hyaluronic acid structures for the polysaccharides. Polysaccharide **1** gave a small, additional peak (**1b**) for polysaccharide, and its high contents of 2-amino-2-deoxyhexose and apparent sulphate are explained by the large amount of protein/glycoprotein/nucleic acid with which it was associated.

Although chondroitin, which contains 2-amino-2-deoxy-D-galactose, and heparan sulphate, which contains 2-amino-2-deoxy-D-glucose, were eluted in the same positions as polysaccharides **2** and **3**, respectively, their absence was proved by the identification of the 2-amino-2-deoxyhexose. Hence, there is a difference between human and rat skin, since the latter is reported to contain heparan sulphate[19].

Polysaccharide **3** is assigned the structure of chondroitin 4-sulphate by the following reasoning. The polysaccharide contains equimolar amounts of hexuronic acid, sulphate, and 2-amino-2-deoxyhexose having the *galacto*-configuration. Total degradation by testicular hyaluronidase indicated the presence of D-glucuronic acid and 2-acetamido-2-deoxy-D-hexose linked β-(1→4) to D-glucuronic acid. That the 2-amino-2-deoxyhexose was substituted at C-4 was demonstrated by its non-participation in the Morgan–Elson assay, since it was subsequently demonstrated by hydrolysis and application of the Elson–Morgan assay that the compound was *N*-acetylated. Total susceptibility to chondroitinase confirmed the presence of D-glucuronic acid and 2-acetamido-2-deoxy-D-hexose linked β-(1→4) to D-glucuronic acid. Since the product of digestion with the chondroitinase–sulphatase preparation was a non-sulphated disaccharide, the sulphate group of the parent polysaccharide is attached to C-4 of the 2-amino-2-deoxyhexose residue.

Polysaccharide **4** is assigned the structure of chondroitin 6-sulphate by similar reasoning, but with the following differences. A positive reaction of the products of hydrolysis with testicular hyaluronidase to the Morgan–Elson assay indicated that the 2-amino-2-deoxyhexose was *N*-acetylated and that the 4-position of the same residue was unsubstituted. The chondroitinase gave a non-sulphated disaccharide, and therefore the sulphate group is located at the 6-position of the 2-amino-2-deoxyhexose residue.

Polysaccharide **5** is assigned the structure of dermatan sulphate by the following reasoning. The polysaccharide contained approximately equimolar proportions of hexuronic acid, sulphate, and 2-amino-2-deoxyhexose. The apparently low value for hexuronic acid could have been due to the presence of protein or of L-iduronic acid. The polysaccharide was completely resistant to testicular hyaluronidase, indicating

the absence of D-glucuronic acid but the presence of L-iduronic acid. Total susceptibility to the chondroitinase demonstrated a β-(1→4)-linkage from 2-acetamido-2-deoxy-D-hexose to hexuronic acid, and confirmed, in conjunction with the foregoing data, that this uronic acid has the L-*ido* configuration. Since the product of chondroitinase–sulphatase hydrolysis was a non-sulphated disaccharide, the sulphate group is located at the 4- or 6-position of the 2-amino-2-deoxyhexose residue. The Elson–Morgan behaviour of the disaccharide demonstrated a (1→3)-linkage from uronic acid to 2-amino-2-deoxyhexose.

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THE IDENTIFICATION OF ACIDIC MUCOPOLYSACCHARIDES FROM HUMAN SKIN BY RADIOACTIVE INCORPORATION

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ABSTRACT

Hyaluronic acids, chondroitin 4- and 6-sulphates, dermatan sulphate, heparin, and keratan sulphate have been identified in human skin by radioactive incorporation of general precursors and by the incorporation patterns related to turnover rates. L-Iduronic acid-*6*-^{14}C, D-glucuronic acid-^{14}C (*U*), and 2-(acetamido-*1*-^{14}C)-2-deoxy-D-galactose were incorporated principally into dermatan sulphate, hyaluronic acid, and the chondroitin sulphates, respectively, whereas the generally low incorporation into keratan sulphate, which has the lowest turnover rate, could be improved by using D-galactose-*6*-^{14}C. The distribution of the polysaccharides in the epidermis and dermis has been investigated, and evidence is presented for the occurrence of over-sulphated chondroitin sulphates in certain tissue.

INTRODUCTION

Acidic mucopolysaccharides can be distinguished by their constituent monosaccharides and sulphate content. An investigation of the specific incorporation of particular precursors is presented.

MATERIALS AND METHODS

Radioactive precursors. — D-Glucose-^{14}C (*U*) (3.00 mCi/mmole), D-galactose-*6*-^{14}C (3.75 mCi/mmole), potassium D-glucuronate-^{14}C (*U*) (2.65 mCi/mmole), and sodium sulphate-^{35}S solution (1.0 mCi/ml, carrier free) were obtained from the Radiochemical Centre, Amersham, Bucks.

L-Idofuranurono-6,3-lactone-*6*-^{14}C was prepared[1] by stirring a mixture of barium 1,2-*O*-isopropylidene-*β*-L-iduronate-*6*-^{14}C (5 mg) and oxalic acid (100 mg) in water (500 *μ*l) for 1.5 h at 100°, decolourising charcoal being added for the last 0.5 h. The filtered, cooled solution was evaporated to a syrup which crystallised. The lactone was converted into the acid by dissolution in 0.1M sodium acetate and was purified[2] by chromatography on Dowex-1 x2 (acetate, 200–400 mesh) resin.

2-(Acetamido-*1*-^{14}C)-2-deoxy-D-glucose and 2-(acetamido-*1*-^{14}C)-2-deoxy-D-galactose were prepared as follows[3]. (Acetic-*1*-^{14}C) anhydride (The Radiochemical Centre, 15 mCi/mmole, 100 μCi), diluted in a vacuum apparatus to 360 μl with acetic anhydride, was distilled at 15° into solutions of 2-amino-2-deoxy-D-glucose or 2-amino-2-deoxy-D-galactose hydrochlorides (300 mg) in water (6.9 ml) and methanol (0.7 ml), containing Dowex-1 x8 (CO_3^{2-}, 20–50 mesh, 8.3 ml) resin. The suspensions were stirred for 2 h at 4°, the filtered solutions were passed through Amberlite IR-120 (H^+, 20–50 mesh, 5 ml) resin, and the boiled eluates were concentrated *in vacuo*. The specific activities of the crystalline products were determined by scintillation counting techniques: 2-(acetamido-*1*-^{14}C)-2-deoxy-D-glucose, m.p. 207°, 4.44 μCi/100 mg; and 2-(acetamido-*1*-^{14}C)-2-deoxy-D-galactose, m.p. 130°, 5.89 μCi/100 mg). The chemical and radiochemical purity of the products was established by paper chromatography with butyl alcohol–pyridine–water[4] (6:4:3); the developed chromatograms were autoradiographed, and the carbohydrates were detected with silver nitrate.

Tissue culture. — Culture medium was prepared from balanced salt solution[5], to which had been added lactalbumin hydrolysate, penicillin, and streptomycin, to give final concentrations of 0.5%, and 120 and 48 units/ml, respectively, and horse serum (3:2 v/v). A solution of the appropriate radioactive compound (1:20 v/v) was added through a Millipore filter.

Human skin ($\geq$ 50 mg, wet weight) was cultured[6,7] at 37° on radioactive media (1 ml) with gentle rocking to ensure equilibration of the aqueous and gaseous (oxygen–carbon dioxide, 19:1) phases. A small portion of the cultured tissue was fixed, and 5-μ sections were stained with haematoxylin and eosin to verify viability during culture. The epidermis and dermis of the larger portion were washed in physiological saline, and separated by treatment with sterilised 0.15% w/v aqueous trypsin for 24 h at 2°. Although trypsinisation at 37° is quicker, it has been shown that this causes greater attack on the other skin constituents besides the cementing substances[8].

Determination of incorporation. — The acidic mucopolysaccharides were extracted and separated by the previously described method[9] on a diminished scale. The dried, homogenised tissue, after being defatted by solvent extraction, was incubated for 24 h at 37° with proteolytic enzyme. The residue from the centrifuged incubate was treated with 0.5N sodium hydroxide for 0.5 h at 37°, neutralised, and centrifuged. The extraction procedure was completed on the combined supernatants by protein denaturation and dialysis.

The polysaccharide extract, concentrated by rotary evaporation *in vacuo* at 37°, was fractionated[9,10] on a column (44.0 × 0.4 cm) of De-Acidite FF (Cl^-, 100–200 mesh) resin, and the dialysed fractions were concentrated to a standard volume (1.0 ml) in glass vials of low potassium content by evaporation in an air stream at 70°. Scintillator fluid (10 ml), consisting of naphthalene (325 g), 2,5-diphenyloxazole (40 g), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (2 g) dissolved in xylene (500 ml), 2-ethoxyethanol (1,500 ml), and *p*-dioxane (1,500 ml), was added. This scintillator system was chosen for its ability to accept high proportions of water and to resist the high quenching normally experienced with aqueous samples. The

radioactive content of each fraction was determined with an automatic liquid scintillation spectrometer.

RESULTS

The extent of incorporation into each polysaccharide is expressed in terms of a percentage of the total incorporation into the polysaccharides of the particular layer of skin. The results obtained for specific precursors are shown in Table I, and the results for the general precursors, D-glucose and sulphate, in Table II. Typical fractionation patterns on De-Acidite FF resin are shown in Fig. 1.

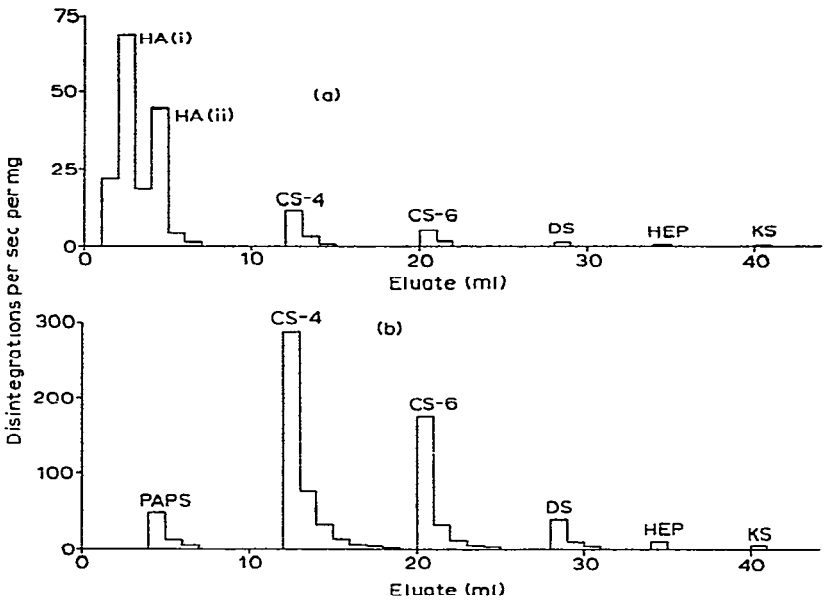


Fig. 1. Incorporation of (*a*) D-glucose-^{14}C (*U*) and (*b*) sulphate-^{35}S into epidermis. HA, hyaluronic acid; CS-4, chondroitin 4-sulphate; CS-6, chondroitin 6-sulphate; DS, dermatan sulphate; HEP, heparin; KS, keratan sulphate; PAPS, adenosine 3-phosphate 5-phosphosulphate.

As a general rule, the total incorporation of radioactivity into the epidermis was twice the dermal incorporation. Since only limited amounts of tissue were available, it was not possible to use tissues from one body site alone, and it was observed that tissue from the ears, as used for the second sulphate-^{35}S figures, gave a more complex pattern. Two additional peaks occurred, these being eluted just after chondroitin 4-sulphate (CS-4') and chondroitin 6-sulphate (CS-6').

DISCUSSION

The structural identification of the principal polysaccharides of human skin has already been performed[9]. The present work indicated the additional presence of

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TABLE I

INCORPORATION OF SPECIFIC PRECURSORS

Precursor	*Culture time* (h)	*Tissue layer*	*Percentage of total incorporation*					
			HA[a]	*CS-4*	*CS-6*	*DS*	*HEP*	*KS*
L-Iduronic acid-6-^{14}C	4	epidermis	0.0	0.0	0.0	100.0	0.0	0.0
		dermis	0.0	0.0	0.0	100.0	0.0	0.0
L-Iduronic acid-6-^{14}C	24	whole skin	14.9	28.2	19.2	37.7	0.0	0.0
D-Glucuronic acid-^{14}C (*U*)	4	epidermis	41.7	21.8	13.4	11.1	11.5	0.5
		dermis	66.7	19.0	5.9	5.6	2.8	0.0
D-Galactose-6-^{14}C	4	epidermis	77.4	9.7	4.7	2.9	2.6	2.7
		dermis	80.3	9.5	6.1	2.7	1.4	0.0
2-(Acetamido-1-^{14}C)-2-deoxy-D-galactose	4	epidermis	6.7	38.4	24.8	20.7	8.7	0.7
		dermis	7.3	45.4	23.2	15.0	9.1	0.0
2-(Acetamido-1-^{14}C)-2-deoxy-D-glucose	4	epidermis	7.9	48.9	21.9	13.9	6.9	0.5
		dermis	5.1	48.4	26.1	12.3	8.1	0.0

[a]See legend to Fig. 1 for key to abbreviations.

TABLE II

INCORPORATION OF GENERAL PRECURSORS

Precursor	*Culture time* (h)	*Tissue layer*	*Percentage of total incorporation*								
			HA(i)[b]	*HA(ii)*	*CS-4*	*CS-4'*	*CS-6*	*CS-6'*	*DS*	*HEP*	*KS*
Sodium sulphate-^{35}S	24	epidermis	0.0	0.0	68.4	0.0	28.5	0.0	2.4	0.6	0.1
		dermis	0.0	0.0	65.1	0.0	27.5	0.0	6.4	1.0	0.0
Sodium sulphate-^{35}S	24	epidermis[a]	0.0	0.0	27.3	10.7	22.4	5.6	20.9	10.8	2.3
		dermis[a]	0.0	0.0	28.8	0.0	22.9	18.8	24.8	4.7	0.0
D-Glucose-^{14}C (*U*)	24	epidermis	58.2	33.9	5.5	0.0	2.2	0.0	0.2	<0.1	<0.1
		dermis	0.0	60.5	21.4	0.0	14.4	0.0	2.9	0.8	0.0

[a]Tissue obtained from ears. [b]Abbreviations as in Table I and text.

heparin, keratan sulphate, and the activated form of sulphate, adenosine 3-phosphate 5-phosphosulphate[10,11]. Furthermore, incorporation of the general precursors, D-glucose and sulphate, differentiated between hyaluronic acid and the sulphated polysaccharides, and reflected the relative rates of turnover of all of the polysaccharides; it was apparent that the rates for heparin and keratan sulphate were particularly low.

During 4-h cultures, L-iduronic acid-*6*-^{14}C was specifically incorporated into dermatan sulphate. Since longer term cultures resulted in incorporation into other polysaccharides, subsequent work was performed with the short time of culture. However, the high percentage incorporation of D-glucuronic acid-^{14}C (*U*) into hyaluronic acid reflected the higher rate of turnover of this polysaccharide; this was not a specific incorporation, since the chondroitin sulphates also contain D-glucuronic acid. These high percentages of incorporation illustrate the use of the precursors for diagnostic work when a particular polysaccharide is being investigated. 2-(Acetamido-*1*-^{14}C)-2-deoxy-D-galactose has a similar use, since it was principally incorporated into the chondroitin sulphates.

Incorporation of these specific precursors confirmed the low rate of turnover of keratan sulphate, but it was evident that the incorporation into this polysaccharide was best achieved by using D-galactose-*6*-^{14}C, one of its constituent monosaccharides.

The preferential incorporation of 2-(acetamido-*1*-^{14}C)-2-deoxy-D-glucose into the chondroitin sulphate and dermatan sulphate was a surprising result. Principal incorporation into hyaluronic acid would have been expected.

Separate investigation of the two skin layers showed that all of the sulphated polysaccharides exist in both the epidermis and dermis, with the possible exception of keratan sulphate in the dermis, since, even in the case of D-galactose-*6*-^{14}C, no peak was evident. The two initial polysaccharide peaks demonstrated by D-glucose-^{14}C (*U*) incorporation are hyaluronic acids; the first contained two separable hyaluronic acid fractions, and the second was homogeneous[9]. The present results show that the degenerate peak arises from the epidermis, whereas the second is present in both layers. The two extra peaks obtained from ear skin, processed by the same techniques, occurred in the elution positions of partially oversulphated chondroitin 4- and 6-sulphates.

These methods of radioactive incorporation should prove useful for investigation of induced effects on mucopolysaccharide synthesis and of clinical conditions.

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FLUOROCARBOHYDRATES

PART XIX[1]. THE MOLECULAR STRUCTURE OF 3,4,6-TRI-*O*-ACETYL-2-BROMO-2-DEOXY-α-D-MANNOPYRANOSYL FLUORIDE AND RELATED COMPOUNDS

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ABSTRACT

In addition reactions between 3,4,6-tri-*O*-acetyl-D-glucal and the elements of "BrF", the major product is 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride (**1**). The structure of this compound has been unequivocally established by X-ray analysis and has been confirmed by ^{19}F magnetic resonance studies. The structures of analogous products, *viz.*, 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-glucopyranosyl fluoride (**2**), 2-bromo-2-deoxy-α-D-galactopyranosyl fluoride (**3**), and 2-bromo-2-deoxy-β-D-arabinopyranosyl fluoride (**4**) have also been confirmed by ^{19}F magnetic resonance studies. The major product from iodofluorination of tri-*O*-acetyl-D-glucal was shown to be 3,4,6-tri-*O*-acetyl-2-deoxy-2-iodo-α-D-mannosyl fluoride (**5**).

INTRODUCTION

Tri-*O*-acetyl-D-glucal is known to react readily with halogens in inert solvents to give 1,2-dihalo products, and in such reactive solvents as methanol to give 2-deoxy-2-haloglycosides[2,3]. Although, in general, the *trans* products predominate, evidence[4] is available from chlorination experiments of four isomeric dihalides, *i.e.*, α- and β-products in the *manno* and *gluco* series. Addition reactions with BrF (anhydrous hydrogen fluoride with *N*-bromosuccinimide[5] or bromine with silver monofluoride[6]) gave principally 3,4,6-tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannosyl fluoride (**1**). Earlier[5], it was suggested that this compound was the β-D-fluoride; the present paper is concerned with the unequivocal establishment of the structure of this compound by X-ray analysis and by magnetic resonance studies.

The findings have been applied to the structures of other 2-haloglycosyl fluorides.

RESULTS

X-ray crystal structure analysis. — Crystal data for the triacetylbromomannosyl fluoride **1** were as follows: $C_{12}H_{16}O_7BrF$ $M = 371.2$, orthorhombic disphenoidal,

TABLE I

ATOMIC PARAMETERS ($\times 10^4$), WITH STANDARD DEVIATIONS ($\times 10^4$) IN PARENTHESIS, FOR THE FLUORIDE **1**

	x/a	y/b	z/c	U_{iso}		x/a	y/b	z/c	U_{iso}
Br	5329 (4)	4875 (2)	−138 (2)	576 (11)	C-5	986 (32)	6405 (14)	−136 (15)	408 (56)
F	1133 (24)	5656 (10)	1583 (10)	721 (44)	C-6	997 (38)	7242 (16)	−171 (17)	517 (69)
O-1	2456 (28)	6459 (11)	481 (11)	596 (47)	C-7	747 (43)	3305 (17)	−414 (18)	636 (82)
O-2	1353 (24)	4065 (10)	−728 (10)	453 (42)	C-8	799 (53)	2588 (20)	−1073 (23)	841 (101)
O-3	−750 (24)	5537 (10)	−1211 (10)	514 (43)	C-9	−766 (43)	5638 (18)	−2186 (17)	599 (72)
O-4	2705 (27)	7228 (11)	−1216 (12)	606 (48)	C-10	−2760 (41)	5462 (18)	−2548 (18)	653 (78)
O-5	−2 (35)	3264 (13)	380 (14)	929 (66)	C-11	2758 (42)	7772 (18)	−1953 (19)	611 (76)
O-6	692 (35)	5828 (13)	−2531 (14)	862 (63)	C-12	4390 (46)	7674 (20)	−2510 (20)	774 (90)
O-7	1494 (34)	8322 (14)	−2016 (14)	851 (62)	H-1	4172	5837	1374	400
C-1	2812 (39)	5758 (16)	1052 (16)	516 (66)	H-2	2711	4302	869	400
C-2	2844 (34)	4873 (16)	466 (15)	510 (60)	H-3	−199	4764	235	400
C-3	1091 (33)	4810 (16)	−162 (16)	488 (59)	H-4	2097	5621	−1245	400
C-4	951 (34)	5583 (14)	−767 (15)	396 (59)	H-5	−302	6337	257	400
					H-6	965	7800	−289	400
					H-7	−205	7253	−1167	400

TABLE II

INTERATOMIC DISTANCES (IN Å) AND INTER-BOND ANGLES (°) WITH STANDARD DEVIATIONS (ESD) FOR FLUORIDE **1**

Br–C-2	1.947 (0.025)	O-6–C-9	1.173 (0.039)	C-1–O-1–C-5	119.0 (2.0)	O-2–C-3–C-4	107.8 (1.9)
F–C-1	1.416 (0.032)	O-7–C-11	1.228 (0.038)	C-3–O-2–C-7	117.9 (2.0)	C-2–C-3–C-4	111.1 (2.0)
O-1–C-1	1.394 (0.033)	C-1–C-2	1.618 (0.037)	C-4–O-3–C-9	118.9 (2.0)	O-3–C-4–C-3	107.6 (1.9)
O-1–C-5	1.373 (0.029)	C-2–C-3	1.536 (0.035)	C-6–O-4–C-11	115.4 (2.1)	O-3–C-4–C-5	109.9 (1.9)
O-2–C-3	1.434 (0.029)	C-3–C-4	1.493 (0.034)	F–C-1–O-1	105.8 (2.0)	C-3–C-4–C-5	107.1 (1.9)
O-2–C-7	1.332 (0.034)	C-4–C-5	1.575 (0.032)	F–C-1–C-2	102.2 (1.9)	O-1–C-5–C-4	116.9 (1.9)
O-3–C-4	1.353 (0.029)	C-5–C-6	1.552 (0.035)	O-1–C-1–C-2	109.9 (2.1)	O-1–C-5–C-6	108.1 (1.9)
O-3–C-9	1.444 (0.035)	C-7–C-8	1.474 (0.047)	Br–C-2–C-1	104.8 (1.6)	C-4–C-5–C-6	110.4 (1.9)
O-4–C-6	1.399 (0.033)	C-9–C-10	1.516 (0.042)	Br–C-2–C-3	115.6 (1.7)	O-4–C-6–C-5	106.3 (2.0)
O-4–C-11	1.373 (0.035)	C-11–C-12	1.411 (0.044)	C-1–C-2–C-3	111.3 (2.0)	O-2–C-7–O-5	119.3 (2.6)
O-5–C-7	1.282 (0.038)			O-2–C-3–C-2	107.4 (1.9)		

$a = 6.97 \pm 0.02$, $b = 15.47 \pm 0.03$, $c = 14.72 \pm 0.03$ Å, $U = 1586$ Å^3, $D_m = 1.54$, $z = 4$, $D_c = 1.555$, F(000) = 752. Space group $P2_12_12_1$ (D_2^4, No. 19) Cu-K_α radiation, $\mu = 42.8$ cm^{-1}, optically biaxial.

A three-dimensional Patterson function, sharpened to point atoms at rest, was calculated from 867 independent reflections. The four bromine atoms of the unit cell were located from their Harker vectors, but it was not possible to determine the positions of any other atoms. A three-dimensional F_0 synthesis, calculated with phases given by the bromine atoms, could not be interpreted readily and so a three-dimensional minimum function was constructed graphically from the Patterson function. This suggested locations for the atoms of the pyranose ring, the fluorine atom on C-1, and the atoms of the C-4 acetoxyl side chain. Re-examination of the F_0 synthesis confirmed all of these positions and gave the locations of the remaining atoms. The atomic parameters were improved by a further F_0 synthesis with the phases computed from all of the atoms.

The space parameters and the isotropic temperature factors for all atoms were refined by the least-squares method with use of a full matrix (85 × 85). Eleven cycles of refinement were computed by using the weighting scheme

$$\sqrt{w} = \sqrt{1/1 + \left(\frac{F_0 - p_2}{p_1}\right)^2}$$

with $p_1 = p_2 = 20$ on the absolute scale. After the seventh cycle of refinement, the hydrogen atoms attached to the ring carbon atoms and to C-6 were placed geometrically and confirmed from a difference synthesis. The final residual (R) was 10.1% for the 867 independent reflections.

The observed structure amplitudes and the calculated structure factors based on the final atomic[7] co-ordinates given in Table I have been deposited in the Radcliffe Library, Oxford. The standard deviations in Table I were calculated from the inverse of the least-squares normal matrix.

Table II lists some interatomic distances and inter-bond angles with their respective standard deviations.

The differences synthesis calculated from the final atomic co-ordinates showed no significant maxima, and confirmed the absence of solvent of crystallisation.

^{19}F and proton magnetic resonance results. — The ^{19}F magnetic resonance spectrum of fluoride **1** was interpreted on the basis of the above structure, and the values $J_{1,F}$ (gem) = 51.0 ±1.0 Hz and $J_{2,F}$ (gauche) = 2.7 ±0.3 Hz were assigned.

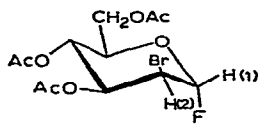
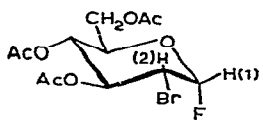
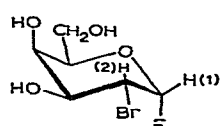
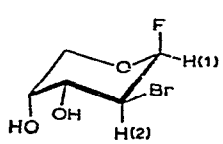
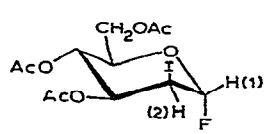
Similarly, values of $J_{1,F} = 51.3 \pm 1.0$ Hz and $J_{2,F} = 27.3 \pm 0.7$ Hz were observed for compound **2**. Since chemical evidence establishes that the bromine atom on C-2 is equatorial and the fluorine atom on C-1 is axial, the value for $J_{2,F}$ is assigned to the *trans*-diaxial arrangement of F and H-2.

The values observed for J_{gauche} and J_{trans} in compounds **1** and **2**, and particularly their difference, may thus provide a simple means of establishing the stereochemistry at C-1–C-2. This has also been noted by Hall and Manville[6,13]

who have concluded that the stereospecificity observed with vicinal ^1H–^1H couplings is paralleled by ^{19}F–^1H couplings. Accordingly, the ^{19}F–^1H coupling constants of three related sugars, 2-bromo-2-deoxy-α-D-galactopyranosyl fluoride[8] (3), 2-bromo-2-deoxy-β-D-arabinopyranosyl fluoride[9] (4), and 3,4,6,tri-*O*-acetyl-2-deoxy-2-iodo-α-D-mannopyranosyl fluoride[10] (5) have been determined, and the results are summarised in Table III.

TABLE III

^{19}F-COUPLING CONSTANTS FOR SOME TRI-*O*-ACETYL-2-DEOXY-2-HALOGLYCOSYL FLUORIDES

	Compound	$J_{1,F}$ (*Hz*)	$J_{2,F}$ (*Hz*)
	1	51.0 ±1.0	2.7 ±0.3
	2	51.3 ±1.0	27.8 ±0.7
	3	52.0 ±1.0	25.0 ±2.0
	4	51.2 ±1.0	25.8 ±1.0
	5	51.0 ±1.0	~1

The n.m.r. evidence provides confirmation of the published structure[8] of compound **3**, but it is clear that the published structure[10] for compound **5** is incorrect in the assignment of configuration at C-1. The value of $J_{2,F}$ for **5** corresponds to a *gauche* arrangement of H-2 and F, as observed for **1** which is now established as the α-D isomer. The value of $J_{2,F}$ for compound **4** is assigned as a *trans* coupling constant,

and it would appear that **4** is thus in the *1C* conformation. Since the *C1* conformer is required for the rapid epoxide formation reported for this compound[9], it must therefore be present in trace amounts. This appears to be confirmed from observation of the ^{19}F spectrum of compound **5**, which provides no detectable change on cooling to $-70°$.

The coupling constants $J_{2,F}$ for the system present in compounds **1**, **2**, **3**, and **5** have also been reported recently by Hall and Manville[13] and differ slightly from those reported here. This may well arise from the use of different solvents[11]. The qualitative conclusions, however, remain unaltered.

DISCUSSION

From the X-ray analysis it is possible to draw a detailed structure for the fluoride **1**. Fig. 1 shows the crystal structure of **1** projected down the *b* axis. Fig. 2 shows the molecule projected (*a*) parallel and (*b*) perpendicular to the least-squares best plane through the atoms of the acetoxyl side-chain at C-4, the fluorine atom, and C-1. It is clear that the bromine atom at C-2 and the fluorine atom at C-1 are both unequivocally axial. Thus, the compound **1** has the α-D-*manno* configuration, and not the β-D-*manno* structure originally suggested. It is also apparent that the three acetoxyl side-chains are planar in the crystal, with the terminal methyl groups *trans* to the ring carbon–side chain bond, and are approximately perpendicular to the pyranose ring. The pyranose ring is in the expected chair conformation.

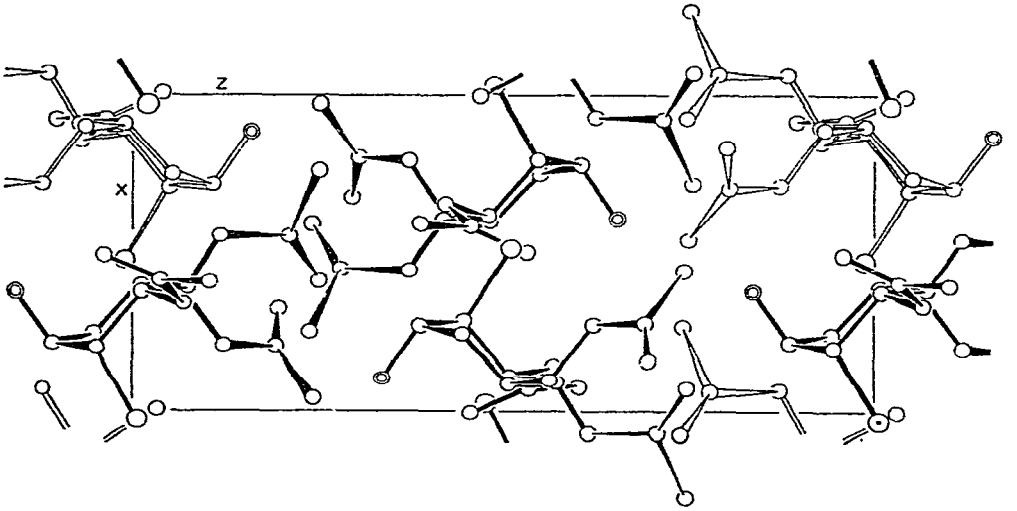


Fig. 1. The molecular arrangements in the fluoride **1**, shown projected down the *b* axis. The molecules drawn with open circles are the furthest away from the observer.

The bond distances are within the expected range, considering the relatively large, standard deviations found. The C-2–Br bond distance of 1.95 Å is similar to

the paraffinic equivalent of 1.94 Å. The C-1–F bond distance of 1.42 Å is slightly greater than the mean value for the paraffinic C–F bond[15] (1.38 Å).

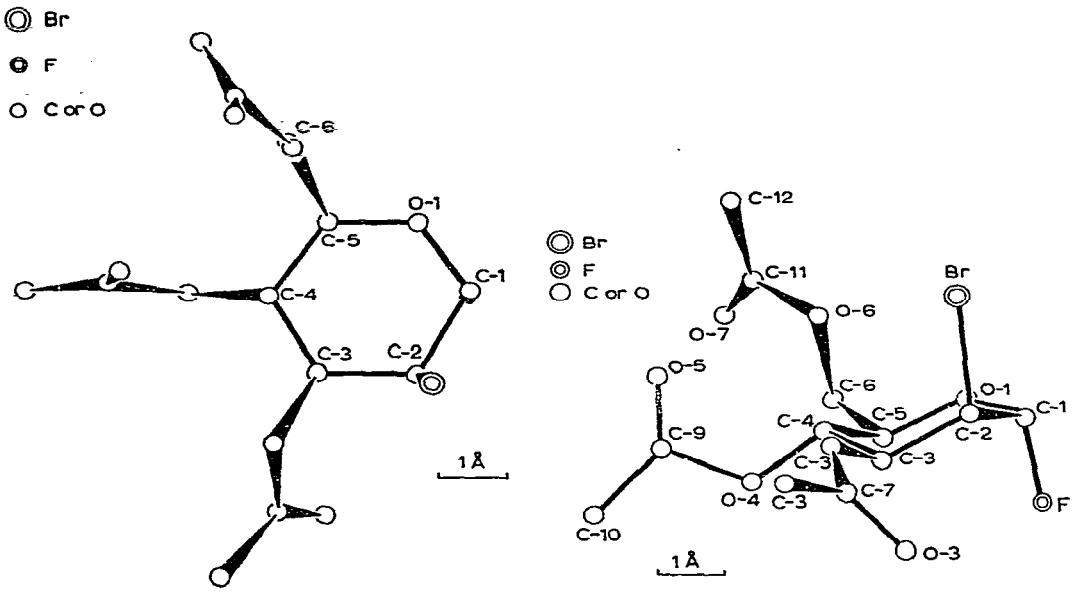


Fig. 2. The fluoride **1** molecule projected (*a*) parallel and (*b*) perpendicular to the least-squares best plane through the atoms F, C-1, C-4, C-9, C-10, O-4, and O-5.

The determination of the absolute conformation of compound **1** thus provides a sound basis from which to conduct magnetic resonance investigations. The ^{19}F chemical shifts and coupling constants are in fair agreement with those determined by Hall and Manville[6,13]. It is clear that the distinctive coupling constants for $J_{2,F}$ (gauche) and (*trans*) provide a simple diagnostic test for establishing the stereochemistry of the C-1–C-2 positions.

These structural determinations indicate that the chief products of BrF and IF addition to tri-*O*-acetyl-D-glucal are not exceptional but resemble those obtained in other halogenations and halomethoxylations, and it is considered that the mechanism of the reaction probably follows a similar course.

A preliminary account of these results has already been published[14].

EXPERIMENTAL

Preparation. — 3,4,6-Tri-*O*-acetyl-2-bromo-2-deoxy-α-D-mannopyranosyl fluoride[5], after repeated recrystallization from 20% (v/v) chloroform–light petroleum (b.p. 60–80°), had m.p. 140.5–141.5°, $[\alpha]_D^{20}$ −30.5° (*c* 1.0, chloroform) (Found; C, 38.7; H, 4.4; Br, 21.5; F, 5.1. $C_{12}H_{16}BrFO_7$ calc.: C, 38.8; H, 4.4; Br, 21.5; F, 5.1%).

X-Ray photography. — The unit-cell dimensions were obtained from calibrated,

zero-layer Weissenberg photographs taken about the principal axes. The intensities of the independent reflections were estimated visually from sets of multiple-film Weissenberg photographs, layers 0-5, taken about the *a* axis. The results from individual layers were placed on a common scale by using the exposure time to *X*-rays from a stable source and standard development conditions. This scaling was modified during refinement by comparison of F_0 and F_c. The intensities were corrected for Lorentz and polarization factors but not for absorption or extinction.

All calculations were computed on an English Electric KDF9 computer by use of programmes developed by Rollett and Hodder[12]. The atomic scattering factors were taken from International Tables for X-ray Crystallography Vol. III.

N.m.r. measurements. — ^{19}F spectra were obtained at 94 MHz on a JEOL-JNM-4H-100 spectrometer operating at 32°, with deuterochloroform as solvent.

ACKNOWLEDGMENTS

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POLYSACCHARIDES ELABORATED BY *Polyporus fomentarius* (Fr.) AND *Polyporus igniarius* (Fr.)

PART I. WATER-SOLUBLE NEUTRAL POLYSACCHARIDES FROM THE FRUIT BODIES

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ABSTRACT

The fruit bodies of the fungi *Polyporus fomentarius* and *Polyporus igniarius* have been examined for polysaccharide content. They both contain a mannofucogalactan and a glucuronoglucan. Investigation of the neutral polysaccharides has shown that they have a similar structure, consisting of a backbone of α-(1→6)-linked D-galactopyranosyl residues, of which 30 to 40% are substituted in the 2-position by L-fucopyranosyl residues, 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl residues, or D-galactopyranosyl residues.

INTRODUCTION

In previous publications, we have reported studies of different polysaccharides isolated from wood-destroying fungi of the Basidiomycetes group, *Polyporus giganteus*[1], *P. pinicola*[2], and *Armillaria mellea*[3]. In the present paper, studies of the polysaccharides from two other fungi, *P. fomentarius* and *P. igniarius*, are reported.

RESULTS

Fruit bodies of *P. fomentarius* and *P. igniarius*, growing on dead birches, were harvested locally. The disintegrated material was extracted, first with hot water and then with 0.3M potassium hydroxide at room temperature, and the extracts were processed to give polysaccharide fractions. Hydrolysates of all four fractions contained D-glucose as the predominant sugar, and smaller amounts of D-galactose, D-mannose, L-fucose, D-xylose (traces only from the material extracted with hot water), and D-glucuronic acid. Because of the similarity of the fractions, only the materials extracted with hot water from the two fungi were further investigated. Neutral and acidic polysaccharides were separated by treatment with DEAE-cellulose, on which the latter were adsorbed and subsequently desorbed by treatment with aqueous potassium acetate. Studies of the polysaccharides, composed of D-glucose and D-glucuronic acid residues, will be reported separately. The neutral fractions still contained considerable proportions of glucans, probably both α-D-glucans and β-D-glucans. Part of the latter were removed by precipitation with cetyltrimethyl-

ammonium hydroxide[4] (CTA-OH). The heteropolysaccharide was then enriched by precipitation with boric acid and CTA-OH and obtained free from glucan by a further precipitation with this reagent.

The polysaccharide from *P. fomentarius* had $[\alpha]_{5780}$ +114° and on hydrolysis yielded D-galactose, D-mannose, and L-fucose in the relative, molar proportions 5.8:1.0:1.2. The corresponding values for the polysaccharide from *P. igniarius* were $[\alpha]_{5780}$ +119° and 6.0:1.0:1.4.

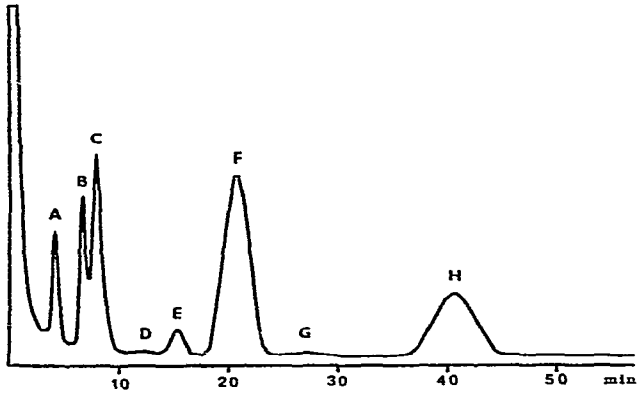
Attempted fractionation of these materials on columns of Sephadex G-150 and Sepharose 4B gave a single peak. Sugar analyses of extreme fractions (*P. fomentarius*) from the fractionation on Sepharose gave D-galactose, D-mannose, and L-fucose, in the relative proportions 5.1:1.0:1.1, from the first-eluted material, and 8.1:1.0:1.1 for the last-eluted material. This indicates some heterogeneity. The possible presence of a contaminating mannan, as indicated by the methylation analyses (see below), could not be demonstrated by these experiments.

The polysaccharides were methylated by two treatments with the methylsulphinyl carbanion and methyl iodide in methyl sulphoxide, following the procedure devised by Sandford and Conrad[5]. The fully methylated polysaccharides were hydrolysed, and the resulting sugars reduced with sodium borohydride to alditols wich were acetylated. The mixtures of partially methylated alditol acetates were then analysed by g.l.c.[6](Fig. 1)–mass spectrometry[7]. The results are summarised in Table I. It has been demonstrated that, for partially methylated alditol acetates, a unique mass-spectrum is obtained for each substitution pattern. Isomeric alditol acetates having identical substitution do not, however, give significantly different mass-spectra. Thus, it was not possible, in the present investigation, to identify the methylated sugars from their mass spectra alone. When, however, the mass-spectrometric evidence was combined with the retention times (*T*-values), the different components could be identified.

The component in peak *A* (Fig. 1) had the same retention time and mass spectrum as the alditol acetate derived from 2,3,4-tri-*O*-methyl-L-fucose. Peaks *B* and *C* were not well resolved, but a better separation, into three peaks, was obtained when g.l.c. was performed at 150° instead of 190°. The fastest component was shown, by its *T*-value and mass spectrum, to be derived from 2,3,4,6-tetra-*O*-methyl-D-mannose, and the slowest was identified, in an analogous way, as being derived from 2,3,4,6-tetra-*O*-methyl-D-galactose. The component having an intermediate *T*-value gave the mass spectrum expected for a 6-deoxy-2,4-di-*O*-methylhexitol acetate (Fig. 2). A reference sample of 2,4-di-*O*-methyl-L-fucose was not available, but, as L-fucose is the only 6-deoxyhexose in the hydrolysate of the polysaccharide, this component must be derived from that sugar.

The minor components in *D* and *G* were not identified. Peak *E* was shown from its mass spectrum to contain comparable amounts of two alditol acetates, derived from a 2,3,4- and a 3,4,6-tri-*O*-methylhexose. The *T*-values for the alditol acetates derived from 2,3,4-tri-*O*-methyl-D-mannose, 3,4,6-tri-*O*-methyl-D-mannose, and 2,3,4-tri-*O*-methyl-D-galactose are 2.48, 1.95, and 3.41, respectively. Only the first

agrees with the *T*-value for the components in peak *E* (2.47). A reference sample of 3,4,6-tri-*O*-methyl-D-galactose was not available, but it seems reasonable to assume that the second component in peak *E* is derived from this substance*.



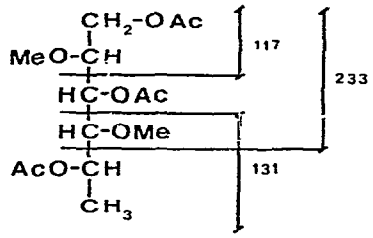


Fig. 1. G.l.c. separation of methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated heterogalactan.

Fig. 2. Primary fragments obtained from 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucose.

The components in peaks *F* and *H* were identified, by their mass spectra and *T*-values, as being derived from 2,3,4-tri-*O*-methyl-D-galactose and 3,4-di-*O*-methyl-D-galactose, respectively. From the results of the methylation analysis, the molar proportions of D-galactose, D-mannose, and L-fucose can be estimated as 5.1:1:1.2 (*P. fomentarius*) and 5.5:1:1.3 (*P. igniarius*), respectively, in reasonably good agreement with the sugar analysis.

Both polysaccharides were subjected to stepwise hydrolysis with acid, and the fragments of low molecular weight were isolated between each step. The monosaccharides were separated and identified as D-mannose, D-galactose, and L-fucose. The main disaccharide component was indistinguishable, by optical rotation, paper chromatography, and electrophoresis, from 3-*O*-α-D-mannopyranosyl-L-fucose, obtained by graded hydrolysis of related, fungal polysaccharides[1,3]. Chromatographic investigation of the products of its hydrolysis, before and after borohydride reduction, gave results in agreement with this structure. The structure is also consistent with the results of the methylation analysis of the polysaccharide, in which equimolecular amounts of 2,4-di-*O*-methyl-L-fucose and 2,3,4,6-tetra-*O*-methyl-D-mannose were formed (Table I).

The polymeric residue after mild hydrolyses had a high optical rotation, $[\alpha]_{5780}$ +176° (*P. fomentarius*) and $[\alpha]_{5780}$ +210° (*P. igniarius*). Methylation analysis of these products showed a decrease in 2,3,4-tri-*O*-methyl-L-fucose, 2,4-di-*O*-methyl-L-fucose, and 2,3,4,6-tetra-*O*-methyl-D-mannose, and a corresponding increase in

*Note added in proof. The *T*-value for the alditol acetate derived from 3,4,6-tri-*O*-methyl-D-galactose has now been determined as 2.50, in agreement with the assumption above.

2,3,4-tri-*O*-methyl-D-galactose. This indicates that 3-*O*-α-D-mannopyranosyl-L-fucose and L-fucose side-chains are attached to the galactan chain.

DISCUSSION

From the structural studies, it is evident that the mannofucogalactans from *P. fomentarius* and *P. igniarius* have similar structures (Fig. 3). They consist of a backbone of α-(1→6)-linked D-galactopyranose residues of which about 30% (*P. fomentarius*) and 40% (*P. igniarius*) are substituted in the 2-position. Most of the substituents are either 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl or L-fucopyranosyl residues. From the optical rotation of the polysaccharide and the anomeric

TABLE I

METHYL ETHERS FROM THE HYDROLYSATES OF METHYLATED HETEROGALACTANS

Sugars	T^a	*Molar proportion* (%)	
		P. fomentarius	P. igniarius
2,3,4-Tri-*O*-methyl-L-fucose	0.65	4.9	5.4
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	11.4	10.5
2,4-Di-*O*-methyl-D-fucose	1.12	11.3	10.6
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	5.1	7.7
Unknown	1.97	1.0	0.6
2,3,4-Tri-*O*-methyl-D-mannose	2.47	4.4	4.0
3,4,6-Tri-*O*-methyl-D-galactose			
2,3,4-Tri-*O*-methyl-D-galactose	3.41	40.5	36.5
Unknown	4.28	0.7	1.9
3,4-Di-*O*-methyl-D-galactose	6.93	20.7	22.8

[a]Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

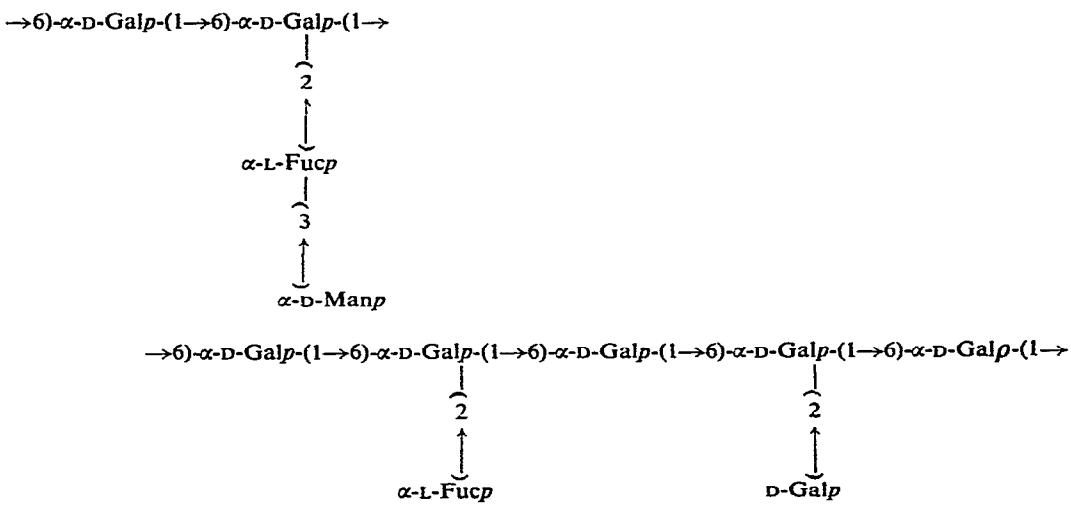


Fig. 3. Proposed structure for heterogalactans from *P. fomentarius* and *P. igniarius*.

structure at the other glycosidic linkages, it may be inferred that the L-fucopyranosyl residues are α-linked. The percentage of 3,4-di-*O*-methyl-D-galactose is higher than the sum of the percentages of 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,4-tri-*O*-methyl-L-fucose, indicating that a considerable part of the 2,3,4,6-tetra-*O*-methyl-D-galactose is also derived from branches. It could not be decided, from these results, if these branches consist of single D-galactose residues, as indicated in Fig. 3, or of longer chains. The structural significance of the 2,3,4-tri-*O*-methyl-D-mannose and the 3,4,6-tri-*O*-methyl-D-galactose is uncertain; the former may be derived from a contaminating mannan. The structures of the polysaccharides are similar to those proposed for other polysaccharides, isolated from other fungi[2,3]. Polysaccharides having this general structure, with minor variations, may therefore be typical for fungi of the Basidiomycetes group.

EXPERIMENTAL

General methods. — Paper chromatograms were run on Whatman No. 1 and 3 MM papers with the following solvent systems (v/v): (*a*) ethyl acetate–acetic acid–water (3:1:1) and (*b*) ethyl acetate–pyridine–water (8:2:1). Components were detected with *p*-anisidine hydrochloride. Paper electrophoresis was performed on Whatman No. 3 MM paper, with 0.05M germanate buffer of pH 10.7 at 40°. Other general methods were the same as in a previous investigation[8].

Isolation of polysaccharides. — The fungus (*P. fomentarius*) (2 kg) was cut into small pieces immediately after harvesting and disintegrated in a Turmix blender in the presence of water. The resulting slurry was extracted with boiling water (2 l) for 1 h, and this procedure was repeated twice. The combined extracts were concentrated and dialysed. A precipitate that formed during the dialysis was removed by centrifugation and discarded, and the polysaccharides (11 g) were precipitated with ethanol. The corresponding fraction from *P. igniarius* (2.1 kg) weighed 12 g.

The fungal material was then extracted, under nitrogen, for 17 h with 0.3M aqueous potassium hydroxide, and this extraction was repeated once. The extract was neutralised with acetic acid, and the polysaccharides (*P. fomentarius*, 15 g; *P. igniarius*, 20 g) were precipitated with ethanol. The fractions, both from the aqueous and alkaline extractions, were dark-brown and obviously contained a high percentage of non-polysaccharide material.

The water-extracted fraction (*P.f.* 11 g, *P. i.* 12 g) was dissolved in water (1.5 l) and DEAE-cellulose (acetate form, 200 g) was added, the mixture was stirred for 2 h, kept overnight, and filtered, and the DEAE-cellulose was washed with water (2 l). The almost colourless solution was concentrated and precipitated with ethanol, to give the neutral polysaccharide fraction (*P.f.* 2.0 g, *P.i.* 2.1 g). The acidic polysaccharides were recovered from the DEAE-cellulose by treatment with 2M aqueous potassium acetate, first in suspension (1.5 l) and then by filtration, until the DEAE-cellulose was almost colourless. The recovered material, which retained all the discolouration, amounted to 4.5 g (*P.f.*) and 8 g (*P.i.*).

The polysaccharide material obtained by alkaline extraction could be separated by the same procedure into a neutral and an acidic fraction.

The neutral polysaccharide from the water extract (*P.f.* 2 g) was dissolved in water (200 ml) and 0.25M aqueous CTA-OH (10 ml) was added. The precipitate formed was separated by centrifugation and treated with acetic acid–ethanol to give a water-soluble fraction (300 mg). Addition of further CTA-OH solution (100 ml) and M sodium hydroxide (4 ml) yielded a second precipitate, which was processed to give 30 mg of polysaccharide. These two fractions on hydrolysis yielded D-glucose only. The supernatant solution was deionised, concentrated, and precipitated with ethanol to give a polysaccharide fraction (1.2 g), which on hydrolysis yielded D-glucose, D-galactose, D-mannose, and L-fucose.

The latter fraction, in 0.1M aqueous boric acid (100 ml), was precipitated with 0.25M CTA-OH (70 ml) and M sodium hydroxide (9 ml), and the precipitate was processed to give a fraction (0.46 g) which on hydrolysis yielded D-galactose, D-mannose, and L-fucose, but only traces of glucose. A fraction (0.5 g) was recovered from the supernatant solution, which on hydrolysis yielded essentially D-glucose. The last traces of D-glucose in the heteropolysaccharide fraction were removed by a second precipitation with the same reagent. The polysaccharide showed $[\alpha]_{5780}$ +114° (*c* 1.1, water).

The neutral fraction from the water extract of *P. igniarius* was worked up by the same procedure to give a heteropolysaccharide fraction (0.2 g), which did not contain D-glucose residues and showed $[\alpha]_{5780}$ +119° (*c* 0.8, water).

For sugar analysis, the heteropolysaccharide (5 mg) was dissolved in 0.25M sulphuric acid (5 ml), the solution was kept for 12 h at 100° and then neutralised with barium carbonate, and the sugars were converted into alditol acetates and analysed by g.l.c.[9]. The identifications of the sugars were confirmed by mass spectrometry[10].

The heteropolysaccharide (100 mg) was placed on a Sepharose 4B column (50 × 0.8 cm). The column was eluted with water, and the fractionation was followed polarimetrically. The extreme fractions (*P. fomentarius*) were subjected to sugar analysis.

Methylation analysis of the polysaccharide. — The polysaccharide (5 mg), in a 5-ml serum bottle sealed with a rubber cap, was dissolved in dry methyl sulphoxide (1.5 ml). Nitrogen gas was flushed through the bottle, and a 2M solution of methylsulphinyl carbanion[5] in methyl sulphoxide (1.0 ml) was added dropwise from a syringe. The resulting, gelatinous solution was agitated in an ultrasonic bath (40 kHz) for 1 h and kept at room temperature for 6 h. Methyl iodide (0.1 ml) was then added dropwise, with external cooling by tap water, and the resulting, turbid solution was agitated for 20 min in the ultrasonic bath, when a clear solution was obtained. A second portion of 2M methylsulphinyl carbanion in methyl sulphoxide (1.0 ml) was added, and the procedure above was repeated, except that an excess of methyl iodide (1.0 ml) was added. The solution was then poured into water (25 ml), dialysed overnight against tap water, and evaporated to dryness.

The methylated polysaccharide was hydrolysed in 0.25M sulphuric acid (3 ml) for 12 h at 100°. The hydrolysate was neutralised with barium carbonate, and the sugars were converted into alditol acetates and analysed by g.l.c.[6]–mass spectrometry[7]. The results are summarised in Table I.

Partial hydrolysis of the heterogalactan. — The polysaccharide (200 mg) was dissolved in 0.025M sulphuric acid (20 ml) and kept for 1 h at 100°. The hydrolysate was neutralised with Dowex-3 (free base) and precipitated with ethanol. The precipitate (100 mg) was dissolved in 0.025M sulphuric acid (10 ml), and the procedure repeated to give a new precipitate (*ca.* 50 mg), which had $[\alpha]_{5780}$ +176° (*c* 1, water) (*P.f.*) and $[\alpha]_{5780}$ +210° (*c* 1, water) (*P.i.*). The precipitate (5 mg) was subjected to methylation analysis. The fragments of low molecular weight from the supernatant solutions were fractionated on thick filter-paper by using solvent system (*b*). The following components were isolated as syrups: D-galactose, $[\alpha]_{5780}$ +72°; D-mannose, $[\alpha]_{5780}$ +10°; L-fucose, $[\alpha]_{5780}$ −54°; 3-*O*-α-D-mannopyranosyl-L-fucose, $[\alpha]_{5780}$ *ca.* 0°. The latter, on hydrolysis, yielded D-mannose and L-fucose. When the hydrolysis was preceded by borohydride reduction, D-mannose was the only reducing sugar observed. The disaccharide was chromatographically [solvent systems (*a*) and (*b*)] and electrophoretically indistinguishable from an authentic sample of 3-*O*-α-D-mannopyranosyl-L-fucose.

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A PROTON MAGNETIC RESONANCE STUDY OF THE ANOMERIC SPECIES PRODUCED BY D-GLUCOSIDASES*

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ABSTRACT

The anomeric configuration of sugars released during enzymic hydrolysis of some D-glucosides and D-glucans in deuterium oxide has been determined by proton magnetic resonance spectroscopy. In some instances, the data are more highly definitive than those provided by methods previously used. The overall results indicate that D-glucosidases and polysaccharases of the *endo* type yield products in which there is retention of the anomeric configuration of the substrate, whereas polysaccharases of the *exo* type cause an inversion of anomeric configuration. It is suggested that these stereochemical differences may originate in differing affinities of the enzymes for various regions of the substrates.

INTRODUCTION

Enzymes that hydrolyze glycosides have long been used for examining the configuration of glycosidic bonds. In a classic study, Armstrong[1] showed that the optical rotation of methyl α- or β-D-glucopyranoside is related directly to the anomeric form (α or β) of D-glucopyranose released when the appropriate, specific enzyme is used. Although these observations helped to establish the configuration of these glycosides, their role was fortuitous, because it was realized only later that the anomer liberated by some D-glycosidases (notably, *beta*-amylase[2]) is the anomerically inverted form, instead of that in which the configuration is retained[3]. The fact that anomeric inversion can take place has become understandable with the finding that, for aldosides, the C-1–glycosidic oxygen bond, not the oxygen–aglycon bond, is cleaved in such hydrolysis[4,5]. Retention of anomeric configuration, then, may be accounted for as being the resultant of two successive anomeric inversions[6].

Such configurational relationships as these have almost invariably been examined by polarimetry. Generally, the results have shown only that the initial products contain a higher proportion of one anomeric species than is present in an equilibrium mixture of the anomers. However, one quantitative treatment has been achieved by

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a kinetic analysis of the release of maltose from amylodextrin by *beta*-amylase[7]. This study demonstrated that only the β anomer of the disaccharide is formed, *i.e.*, that there is quantitative, anomeric inversion. A different approach has recently been described by Parrish and Reese[8], who have shown that gas-chromatographic examination of the reducing sugar liberated may, in some instances, yield quantitative data more reliable than those obtainable by polarimetry.

It occurred to us that proton magnetic resonance (p.m.r.) spectroscopy should also be suitable for examining stereochemical problems of this kind. The chemical shifts and spacings of anomeric protons are usually highly characteristic[9,10] and, therefore, under satisfactory conditions, it should be feasible to detect directly, and characterize, the appropriate signal for the sugar released on enzymic cleavage of a glycosidic bond. The present paper describes several experiments performed in examining this possibility. The systems described have been chosen mainly because of the availability to us of these particular enzymes and substrates in a reasonably pure state.

Experimentally, the procedure consisted simply of admixing enzyme and substrate in deuterium oxide*, or in a buffered deuterium oxide solution, and periodically scanning selected regions of the p.m.r. spectrum recorded by means of a 100-MHz n.m.r. spectrometer. Generally, the signal produced by the anomeric proton of the reducing sugar newly formed was clearly evident as a sharp doublet, although, occasionally, it was obscured by signals due to the parent glycosyl group, the aglycon, or the HOD of the solvent. Interference caused by the last was minimized by preliminary deuterium-exchange. However, this treatment sometimes caused extensive deactivation of an enzyme; alternatively, the HOD signal was displaced slightly upfield or downfield by slightly raising or lowering the temperature of the sample. It should also be noted that, in order to obtain proton signals of sufficient intensity, experiments on polysaccharides have been confined to highly soluble substrates (*i.e.*, at a concentration of about 10%).

As is shown by the accompanying partial p.m.r. spectra (Figs. 1, 2, and 3), it has, in a number of instances, been possible to detect the appearance of one reducing anomer in substantial yield before the presence of the other anomer is at all evident. For example, this effect is seen in Fig. 1a, which shows the low-field side of typical spectra recorded in examining the action of an *exo*-β-D-(1→3)-glucanase (*Basidiomycete* sp. QM806) on laminaran. During early stages of the reaction (*e.g.*, at 975 sec), a strong doublet attributable to H-1 of α-D-glucose is found (at 4.3 p.p.m.; spacing, 3.2 Hz), but there is little indication of an H-1 signal for the β-D anomer. Only later (*e.g.*, at 180 min) is a signal that is due to the latter detected (at 4.9 p.p.m.; spacing, 7.2 Hz), undoubtedly as a result of mutarotation of the α-D anomer.

Similarly, the onset of the hydrolysis of starch by *beta*-amylase is accompanied by the appearance of a strong signal at 4.9 p.p.m. (spacing, 7.2 Hz), attributable to

*It has been assumed that possible deuterium isotope-effects have no bearing on the stereochemistry of these reactions.

H-1 of the β-D anomer of the reducing end-unit of the maltose released, whereas only after a prolonged period is the α-D anomer detected (doublet of 3.2 Hz spacing at 4.3 p.p.m.). Fig. 2b provides evidence that the only sugar present after the hydrolysis of salicin by emulsin is β-D glucose (broad H-1 signal at 4.9 p.p.m.); and Fig. 3a shows that only α-D-glucose (or α-oligosaccharides) is released during the attack of *alpha*-amylase on starch. The other Figures also show that each enzymolysis releases one reducing form in great preponderance over its anomer, although, probably because of accompanying rapid mutarotation, the distinctions are not quite so clear-cut in these experiments.

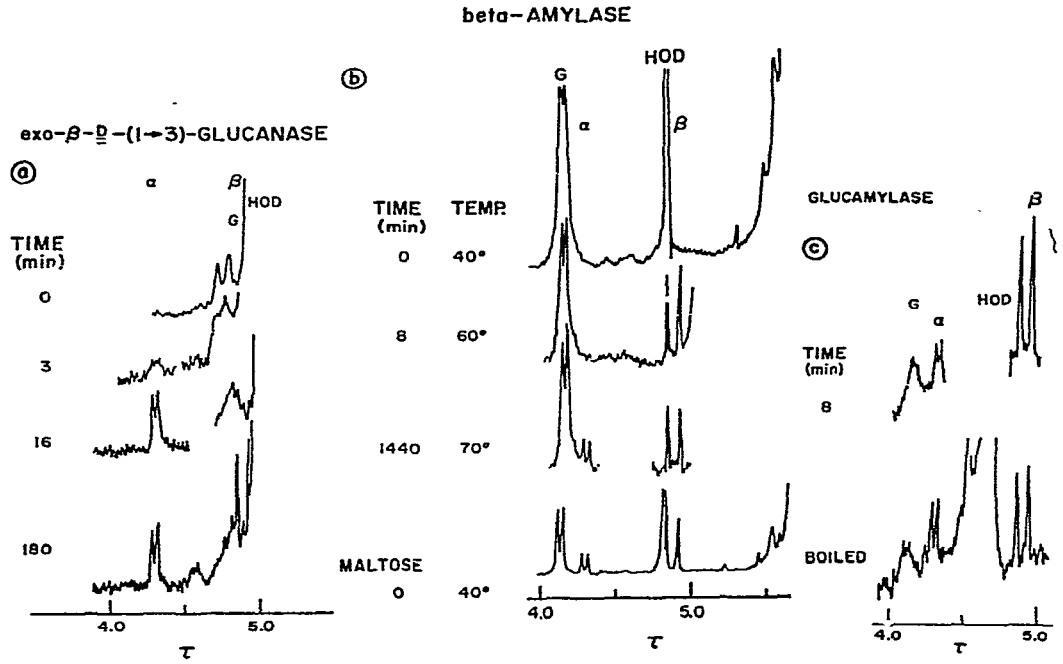


Fig. 1. Partial p.m.r. spectra in deuterium oxide, recorded during assay of the *exo*-glycosidases. (a) *exo*-β-D-(1→3)-Glucanase on laminaran at 45°. α-D-Glucose is produced, and only later is it seen to mutarotate to the β-D anomer. (b) *beta*-Amylase on starch. β-Maltose is seen to be the only product after 8 min. Recording at 60° prevents masking of the H-1-β signals by the peak for residual water. (c) Glucamylase on starch. A predominant H-1-β signal is given after 7 min. The HOD signal is due to residual water; the α-signal is due to H-1 of the α-D anomer released; the β-signal is due to H-1 of the β-D anomer released; the G signal is due to H-1 of the glycosyl unit of the starting substrate.

In at least some of these reactions, then, it appears that one anomeric species is produced exclusively during the initial stages, and that the other is formed subsequently by mutarotation. Although such a high degree of stereospecificity is, perhaps, not surprising, it need not be assumed *a priori* that production of only one anomer is possible, because relatively little is known about the mechanisms of these reactions (see later).

The steric results obtained with the various systems examined are listed in Table I. These results reinforce the general view that an *exo* type of polyglycosidase

(which attacks the polymer at the chain end) produces a sugar having an anomeric configuration opposite to that of the parent glycosyl group, whereas an *endo* type (which attacks randomly), or an enzyme that hydrolyzes a simple glycoside, acts with retention of configuration (see Figs. 2 and 3)*. The feasibility of using these, and other, characteristics for classifying carbohydrases has been discussed recently by Reese *et al.*[11].

TABLE I

EFFECT OF VARIOUS ENZYMES ON SUBSTRATES

Enzyme	*Substrate*	*Configuration of proton released*	*Inversion (I) or retention (R) of anomeric configuration*
Glucosidases			
α-D-Glucosidase (*Saccharomyces cerevesiae*)	phenyl α-D-glucopyranoside	α	R
β-D-Glucosidase (almond)	salicin	β	R
***exo*-Splitting enzymes**			
Glucamylase (*Aspergillus niger*)	starch	β	I
exo-Laminaranase (*Basidiomycete* sp. QM806)	laminaran	α	I
beta-Amylase (sweet potato)	starch	β	I
***endo*-Splitting enzymes**			
alpha-Amylase	starch	α	R
endo-Laminaranase (*Rhizopus arrhizus* QM1032)	laminaran	β	R
endo-Laminaranase (*Cytophaga* sp.)	laminaran	β	R

By use of the above generalization, we have been able to infer, from p.m.r. spectral examination, the presence of an *exo*-laminaranase in a crude-enzyme system capable of degrading the cell walls of *Pythium* sp. This lytic complex shows *endo*-laminaranase activity, since it produces oligosaccharides from laminaran. However, the p.m.r. spectrum of the reaction mixture suggested that α-anomeric residues were being liberated simultaneously with the expected β-form of the oligosaccharides, and hence, that an *exo*-laminaranase might also be present. This conclusion followed from the observations that two H-1 signals were produced and that the relative

*Fungal *endo*-dextranases are apparent exceptions to these generalizations[14].

intensity of the $H_e(\alpha)$ signal was greater than could be accounted for by mutarotation of the β compound. Subsequently, in fact, an *exo*-laminaranase has been isolated from the crude enzyme system[12].

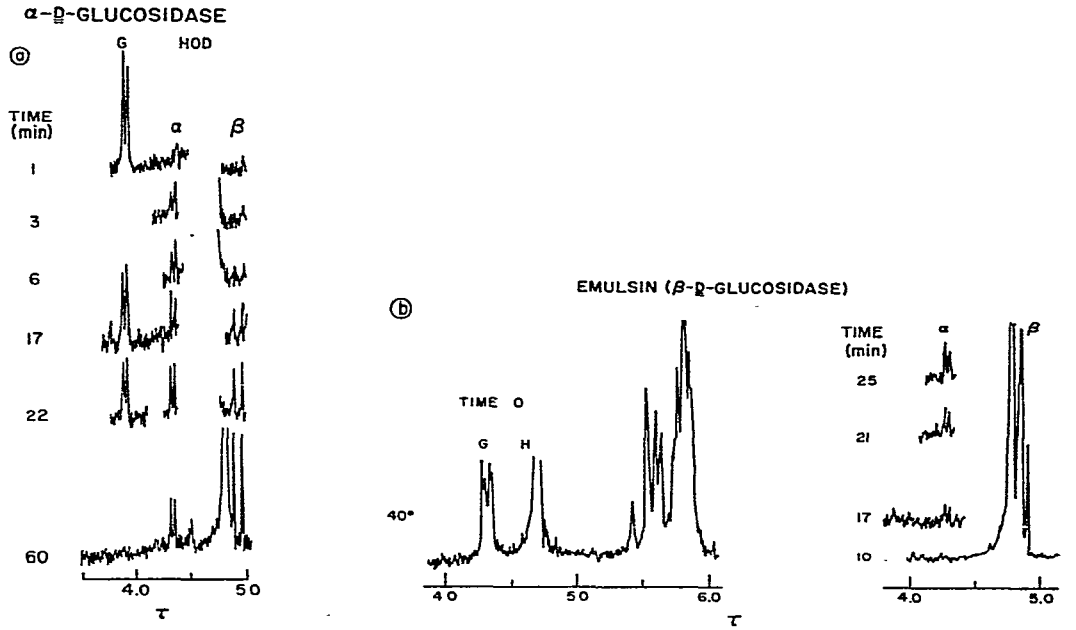


Fig. 2. Partial p.m.r. spectra in deuterium oxide, recorded during assay of the D-glucosidases. (a) α-D-Glucosidase on phenyl α-D-glucopyranoside. α-D-Glucose is seen as the reaction product. (b) β-D-Glucosidase on salicin. β-D-Glucose is seen as the reaction product (10 min) followed by slow inversion. The HOD signal is due to residual water; the α-signal is due to H-1 of the α-D anomer released; the β-signal is due to H-1 of the β-D anomer released; the G signal is due to H-1 of the glycosyl unit of the starting substrate.

Another aspect of these enzymic reactions merits comment. In the enzymic hydrolysis of simple glycosides, the glycosyl moiety is the specific substrate, whereas the structure of the aglycon may be varied widely[13]. The *endo*-laminaranase (see Fig. 3 and Table I), which, like the glycosidases, acts with retention of configuration, may show a similar characteristic. Thus, it is known to show high specificity for the glycosyl moiety (in this instance, for a 3-substituted β-D-glucopyranosyl unit of polysaccharides), but less for the adjacent residue, corresponding to the aglycon of the simple glycoside; that is, the latter residue may be bound[15] either through O-3 or O-4.

exo-Glucanases differ from these types of enzymes, not only in causing inversion, but probably also in having a different substrate requirement. Thus, they appear to show high specificity both for the glycosyl residue that is released and for its "aglycon" (*i.e.*, the residue with which it forms the linkage that is hydrolyzed). There is evidence, for example, that *exo*-laminaranases are specific for cleaving a dimeric 3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl structure[16]; *exo*-cellulase, for the corresponding

4-substituted structures[17]; and *beta*-amylase, for a 4-*O*-α-D-maltosyl-α-D-glucopyranosyl structure[18].

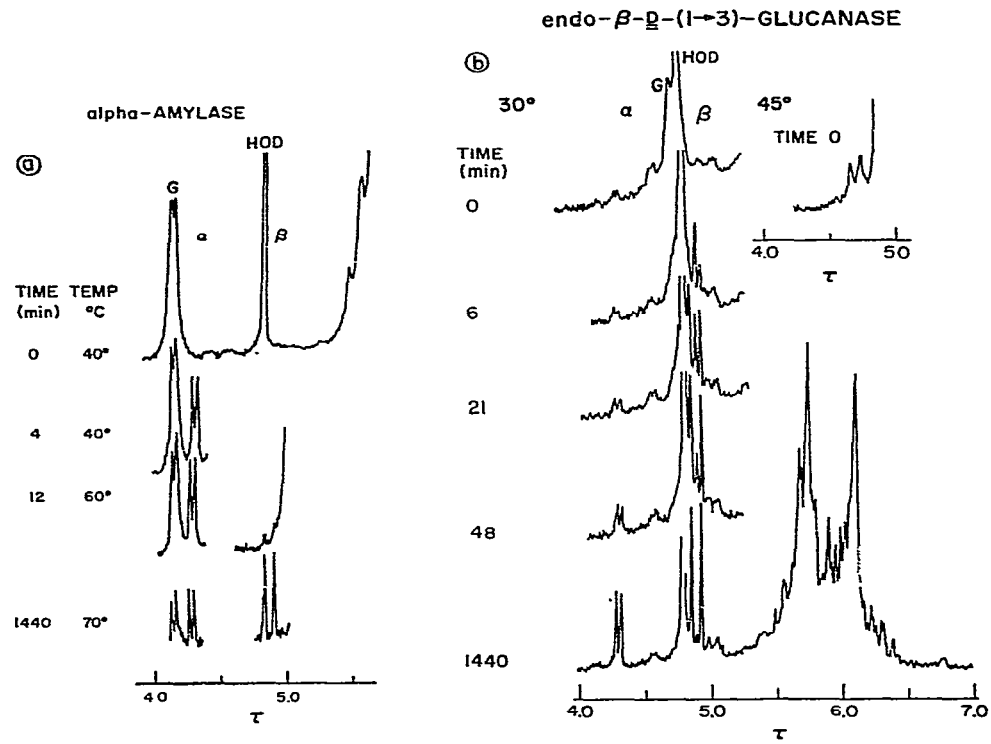


Fig. 3. Partial p.m.r. spectra in deuterium oxide, recorded during assay of the *endo*-glycosidases. (a) *alpha*-Amylase on starch. The H-1-α signals appear initially (4 min), and inversion occurs relatively slowly. (*b*) *endo*-β-D-(1→3)-Glucanase on laminaran. The β-D anomers are seen to be the initial products of the reaction. The HOD signal is due to residual water; the α-signal is due to H-1 of the α-D anomer released; the β-signal is due to H-1 of the β-D anomer released; the G signal is due to H-1 of the glycosyl unit of the starting substrate.

The mechanism of hydrolysis advanced by Mayer and Larner[5] to account for inversion or retention by *alpha*- or *beta*-amylase, respectively, involves different conformations of an intermediate, C-1 carbonium ion generated when the D-glucopyranosyl group is liberated. This type of intermediate could hypothetically lead to the formation of either anomer, or both, depending on how freely it exists and how it is attacked by a molecule of water. Because the data now available indicate that only one anomer is produced on hydrolysis, a more highly stereospecific kind of pathway must be envisaged.

Inversion by an *exo*-glucanase may be depicted, for example, as involving strong association both with (*a*) the glycosyl residue and (*b*) the adjacent residue (OR) to which C-1 is bonded in such a way that the enzyme is suitably disposed for assisting in the departure of the OR portion, *e.g.*, by providing a suitably positioned electrophilic group (as in Fig. 4a). Cleavage of the C-1–glycosidic oxygen bond may then

be accompanied by backside attack of water at C-1, yielding the anomer in which the hydroxyl group is on the opposite side of C-1.

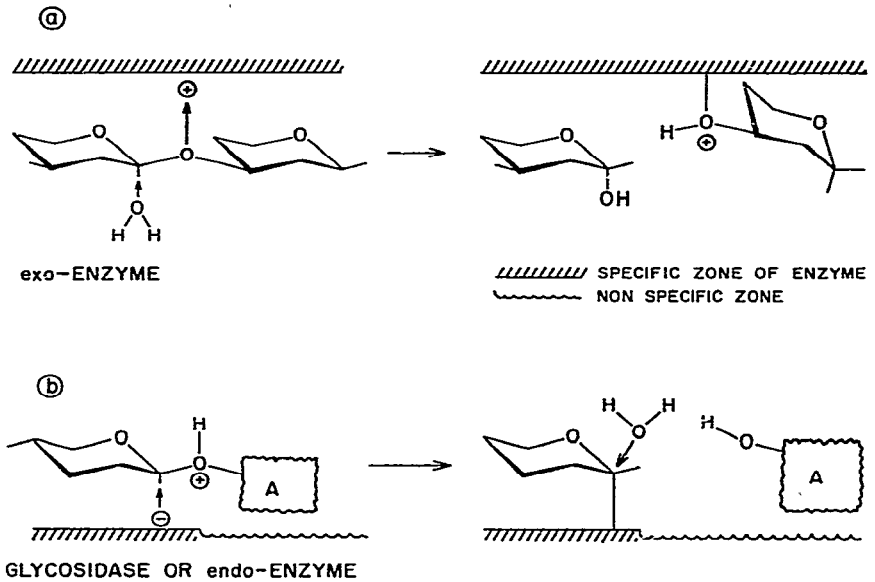


Fig. 4. Representation of reactions between glycosides (including polysaccharides) and hydrolytic enzymes. A is the aglycon or an adjacent residue.

By contrast, retention of anomeric configuration in the product could be the resultant of two consecutive inversions, as has been suggested by Koshland[3]. A nucleophilic group of the enzyme, such as a carboxyl anion, attacks C-1 to yield a glycosyl–enzyme product* of inverted anomeric configuration (as in Fig. 4b). Alternatively, a transient glycosyl carbonium ion could be generated[5], and be stabilized electrostatically by an enzyme COO^- group situated at the side remote from that of the departing OR group (as depicted for lysozyme[20]). In either event, the direction of the subsequent approach by water to C-1 of the glycosyl unit is such as to lead to overall retention of anomeric configuration. Such a pathway shows less obvious need for participation of the OR moiety than does that already envisaged for the *exo*-glycanases, a conclusion in harmony with the specificity characteristics of glycosidases and, possibly, also of *endo*-polyglycosidases.

MATERIALS AND METHODS

Enzymes. — *A.* exo-*Splitting glucanases.* 1. *exo*-α-Glucanase [glucamylase; α-D-(1→4)-glucan 4-glucohydrolase: E.C. 3.2.1.3] from *Aspergillus niger* was "diazyme"

*Legler[19] has recently presented evidence that reaction between a β-D-glucosidase and conduritol β-epoxide (a substrate deactivator) leads to formation of an ester between a carboxyl group of the enzymes and the (+)-inositol resulting from epoxide ring-opening.

(Miles Chemical Co., Elkhart, Indiana). The crude enzyme (5 g; 3.2 DU*/g) was mixed with 20 ml of water, and the suspension was centrifuged to remove the large proportion of insoluble matter. Dialysis of the supernatant liquor produced a loss in dry weight of the enzyme preparation of 70%. Samples containing 20 mg of soluble matter were lyophilized.

2. *beta*-Amylase [α-D-(1→4)-glucan maltohydrolase: E.C. 3.2.1.2]. Sweet-potato *beta*-amylase (WBC**; 25 mg; 700 WU*/mg) was dialyzed against distilled water for 24 h. The preparation was centrifuged, and the supernatant liquor was lyophilized.

3. *exo*-Laminaranase [β-D-(1→3)-glucan glucanohydrolase: E.C. 3.2.1.39] was obtained from[21] *Basidiomycete* sp. QM806. A purified preparation (100 U, specific activity*** 84.5) was dialyzed and lyophilized.

4. *exo*-Laminaranase was obtained from *Streptomyces* sp. 0143 as a purified preparation[12]. A sample containing 300 U was lyophilized.

B. Glucosidases. 1. α-D-Glucosidase (α-D-glucoside glucohydrolase: E.C. 3.2.1.20) was obtained from *Saccharomyces cerevisiae* PRL 324; the enzyme was induced in the presence of maltose. Crude, intracellular enzyme (12 U) was dialyzed and lyophilized.

2. β-D-Glucosidase [β-D-glucoside glucohydrolase: E.C. 3.2.1.21] was obtained from almond (WBC). The enzyme (410 U; specific activity 2.72, with salicin as the substrate) was deuterated with deuterium oxide, and lyophilized; the process was repeated.

C. endo-*Splitting glucanases.* 1. α-Amylase [α-D-(1→4)-glucan 4-glucanohydrolase: E.C. 3.2.1.1.] was obtained from hog pancrease (WBC). The preparation (33 mg, minimal activity 500 WU/mg) was dialyzed and then lyophilized.

2. *endo*-β-D-(1→3)-glucanase [β-D-(1→3 or 4)-glucan glucanohydrolase: E.C. 3.2.1.6]. Three kinds were used. (a) *Rhizopus arrhizus*. The crude enzyme was obtained by precipitation with acetone[21], and 17.3 U was dialyzed and lyophilized. (b) *Cytophaga* sp.[22]. The crude enzyme was received from R. S. C. Aytoun (Glaxo Laboratories, Stoke Poges, Bucks., England). The crude preparation (325 mg, 16.9 U) was dissolved in water, and the solution was dialyzed and then lyophilized. (c) *Streptomyces albidoflavus* crude enzyme[12]. Mixed *endo*- and *exo*-β-D-(1→3)-glucanase (9.63 U) was dialyzed and then lyophilized.

After the various preparative procedures had been carried out, each lyophilized enzyme was treated with deuterium oxide and relyophilized.

Substrates. — Soluble starch (Fisher Chemical Co., Fairhaven, N. J., U. S. A.) that had been reduced with sodium borohydride was used in the *alpha*- and *beta*-

*An enzyme unit (U) is defined as (*a*) that amount of enzyme liberating 1 μmole of reducing groups per min, or (*b*), in the case of commercial preparations, a figure given by the manufacturer (*e.g.*, DU; WU). The degree of inactivation caused by freezing and lyophilization of the enzyme preparations was not determined.

**WBC, Worthington Biochemical Corporation, Freehold, N. J., U. S. A.

***Specific activity is defined as U/mg of protein.

amylase assays. Amylose (50 mg), obtained by partial degradation of starch by[21] *Basidiomycete* sp. QM806, was used in the glucamylase assay. Soluble laminaran (ex *Laminaria cloustoni*) was used for all β-D-(1→3)-glucanase assays. A saturated solution of salicin was used as the substrate for β-D-glucosidase, and of phenyl α-D-glucopyranoside in the α-D-glucosidase assay.

P.m.r. spectroscopic assays. — P.m.r. spectroscopy (with a Varian HA-100 high-resolution n.m.r. spectrometer) was used for establishing the anomeric configuration of the sugars released during enzymic hydrolysis, by detection of the H-1 signal of the sugar liberated. In a typical experiment, the reaction substrate (40 mg) was dissolved in 400 μl of deuterium oxide, and the solution was added to the appropriate, lyophilized enzyme. After dissolution of the enzyme, the reaction mixture was transferred to a standard, n.m.r. spectrometer tube, and the spectrum was recorded periodically, with use of a capillary of tetramethylsilane as the external, reference lock-signal. Most of the reactions were performed at 33°, but, in some instances, the temperature was 45 or 25° (to displace the HOD signal upfield, or downfield, from the H-1 signals being examined).

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ANOMERIC FORMS OF 9-(2-AMINO-2-DEOXY-D-XYLOFURANOSYL)ADENINE

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ABSTRACT

The ethyl 2-acetamido-2-deoxy-1-thio-α-D-xylofuranoside (**1a**) previously reported was further acetylated to the 3,5-diacetate (**1b**), and this was completely deacetylated by successive treatment with phosphorus pentasulfide and methanolic ammonia. After introduction of the *N*-(2,4-dinitrophenyl) group, replacement of the ethylthio group by chlorine yielded the glycosyl chloride derivative **6**, which gave, on treatment with *N*-acetyl(chloromercuri)adenine and removal of all protecting groups, a crystalline mixture (**9a**) of the anomers of 9-(2-amino-2-deoxy-D-xylofuranosyl)adenine. Separation of the anomers on an ion-exchange column gave the pure, crystalline anomers (**9b** and **9c**). Evidence is presented that the anomeric mixture **9a** is a 1:1 molecular compound. Anomeric assignments were made on the basis of n.m.r. spectroscopic and polarimetric data, including circular dichroism.

DISCUSSION

Ethyl 2-acetamido-2-deoxy-1-thio-α-D-xylofuranoside[1-3] (**1a**) was further acetylated to the crystalline 3,5-diacetate (**1b**). The peracetylated 1-thioglycoside **1b** was then completely deacetylated by the successive action of phosphorus pentasulfide and methanolic ammonia, according to the general procedure of Fox and co-workers[4], without crystallization of the intermediates **2** and **3**. The 2,4-dinitrophenyl (DNP) group[5,6] was then introduced onto the nitrogen atom of ethyl 2-amino-2-deoxy-1-thio-α-D-xylofuranoside (**3**), and the syrupy **4** resulting was acetylated to give syrupy ethyl 3,5-di-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-1-thio-α-D-xylofuranoside (**5**) in a form apparently homogeneous (by chromatography). The ethylthio group of **5** was then replaced by chlorine[7], and the resultant, syrupy glycosyl chloride (**6**) was allowed to react with *N*-acetyl(chloromercuri)adenine, according to the general procedure of Davoll and Lowy[8]. The reaction product (**7**), isolated by t.l.c., formed a crystalline picrate on deacetylation with picric acid[9], and this picrate yielded a crystalline mixture (**9a**), $[\alpha]_D^{24}$ -17 $\pm 3°$ (methanol), of the anomers of 9-(2-amino-2-deoxy-D-xylofuranosyl)adenine.

The mixture **9a** was separated into its components on an ion-exchange column, according to the general method of Dekker[10]. The β-D anomer (**9c**), $[\alpha]_D^{24}$ -56 $\pm 2°$

(methanol), crystallized spontaneously. On long standing, the α-D anomer crystallized as a trihydrate, which readily lost its water of hydration to give crystalline, anhydrous 9-(2-amino-2-deoxy-α-D-xylofuranosyl)adenine (**9b**), $[\alpha]_D^{22}$ +23 ±3° (methanol). These optical rotations, although not precisely defined because of the small amount of pure material at hand, would seem to indicate a 1:1 mixture, since $(-56)\beta+(+23)(1-\beta) = -17$; and, therefore, $\beta = 0.5$. The X-ray powder diffraction data of the α,β-D form, in comparison with those of its components, conclusively prove that the α,β form is a definite, molecular compound, of tentative composition 1:1. This conclusion is supported by the n.m.r. spectrum of this material, which showed signals of two anomeric protons of equal intensity. Such a formulation is in agreement with a previous finding that 9-(2-amino-2-deoxy-α,β-D-ribofuranosyl)adenine is a 1:1 molecular compound[11].

Anomeric assignments were first made on the basis of the observed optical rotations. Although the Hudson isorotation rules[12] were never intended to be applied to nucleosides and other glycosylamines, they nevertheless do hold quite well[13] for the substituents that Hudson employed. With such unusual substituents as the 2,4-dinitroanilino group[14], especially on C-2, or the 2,3-epoxy group[15], the optical rotations of the anomers can be reversed. The same holds if no substituent is present on C-2, as in pyrimidine nucleosides of 2-deoxy-D-*erythro*-pentose[16]. The variation of observed circular dichroism with wavelength (see Fig. 1) for each anomer shows a positive Cotton-effect for the α-D anomer (maximum at 260 nm), and a negative effect for the β-D form (maximum at 259 nm), as is required for the anomers of purine nucleosides[17,18].

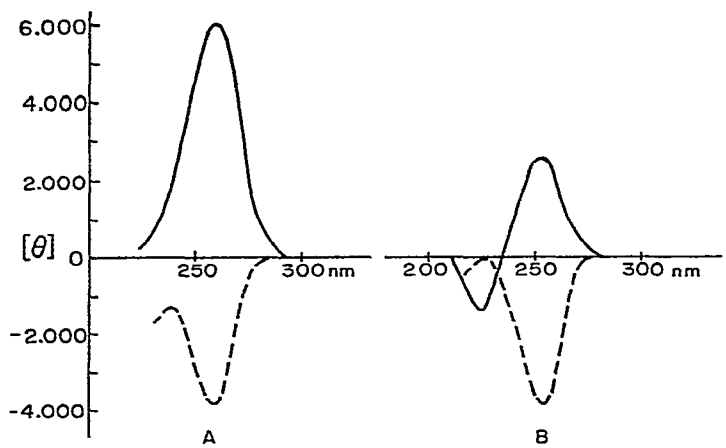
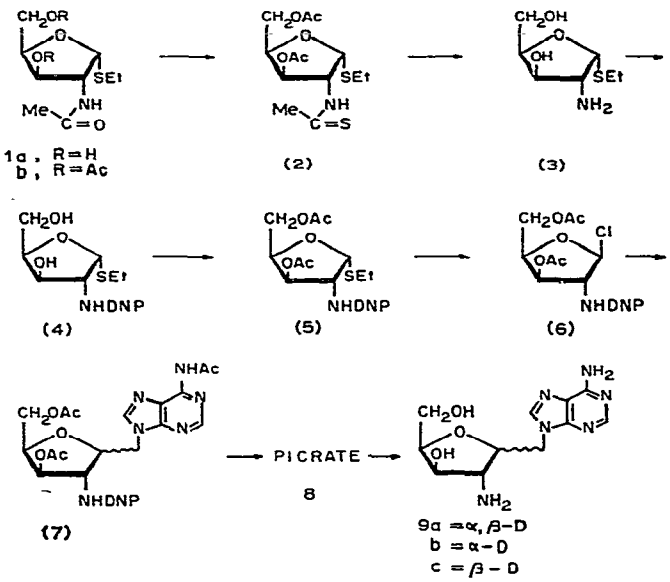


Fig. 1. Circular dichroism curves obtained for the anomers of 9-(2-amino-2-deoxy-D-xylofuranosyl)-adenine with a JASCO Model ORD/UV-5 spectrometer (Japan Spectroscopic Co., Ltd.); —, α-D anomer; ..., β-D anomer; A, in aqueous solution; B, in 0.05 N hydrochloric acid.

The comparative n.m.r. data for the α-D, β-D, and α,β-D forms were complicated, somewhat, because the low solubility of the α-D anomer in deuterium oxide precluded measurement in this solvent. Satisfactory data for both anomers were obtained in

1:1 deuterium oxide–pyridine-d_5. The n.m.r. data for the α,β-D compound clearly show that the anomers are present in equal amounts, and the data for each individual component demonstrate the absence of the other anomer. It has been shown[19] that the signals of the C-1′ protons of pyrimidine 1′,2′-*cis*-nucleosides appear at lower fields than do those of the 1′,2′-*trans* forms. The same had been found for anomeric



pairs of purine nucleosides[11,20,21]. In accordance with this generalization, the anomeric proton of 9-(2-amino-2-deoxy-α-D-xylofuranosyl)adenine (**9b**, *cis*-nucleoside) resonated at lower field than that of the β-D form (**9c**, *trans*-nucleoside).

EXPERIMENTAL

General methods. — Melting points were determined with a Hershberg type of apparatus. Specific rotations were determined in a 2-dm polarimeter tube. I.r. spectra were recorded with a Perkin–Elmer Infracord spectrophotometer. U.v. spectra were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer or with a Jasco spectrophotometer (Japan Spectroscopic Co., Ltd.). N.m.r. data were recorded, in part, by J. D. Wander and J. B. Hughes with a Varian A-60 n.m.r. spectrometer, and, unless otherwise noted, were observed for solutions (freshly prepared) in deuterium oxide or chloroform-*d*, with an internal standard of sodium 4,4-dimethyl-4-silapentane-1-sulfonate or tetramethylsilane, respectively. Microanalyses were made by W. N. Rond. X-Ray powder diffraction data give interplanar spacings, Å, for CuKα radiation, λ 1.539 Å, nickel filter, camera diameter 114.6 mm, with photographic recording. Relative intensities were estimated visually: m, moderate; s, strong; v, very; w, weak. The strongest lines are numbered (1, strongest); multiple numbers indicate approximately equal intensities. T.l.c. (0.25 nm thickness, unless otherwise noted) was performed by the ascending method with Desaga equipment, by use of

Silica Gel G (E. Merck, Darmstadt, Germany), activated at 110°, with detection of colorless materials by sulfuric acid. Indicated amounts of developer are by volume. Unless otherwise noted, evaporations were performed under diminished pressure at 40° or below.

Ethyl 2-acetamido-3,5-di-O-acetyl-2-deoxy-1-thio-α-D-xylofuranoside (**1b**). — Ethyl 2-acetamido-2-deoxy-1-thio-α-D-xylofuranoside[1-3] (**1a**, 5.00 g) was acetylated overnight at room temperature with acetic anhydride (50 ml) and pyridine (50 ml). The reaction mixture was poured into ice and water, the solution was extracted with chloroform, and the extract was washed successively with water, saturated aqueous sodium hydrogen carbonate, and water. The dried (magnesium sulfate) solution was concentrated to a small volume, and then coevaporated with toluene. The residue was crystallized from ethyl acetate–ether–hexane; yield 5.46 g (81%), m.p. 116–117°. Pure material was obtained on recrystallization effected in the same way, m.p. 117–118°, $[\alpha]_D^{23}$ +185 ±2° (*c* 1.46, methanol); λ_{max}^{KBr} 3.08 (NH), 5.75 (OAc), 6.03, and 6.53 μm (NHAc); X-ray powder diffraction data: 12.99 m, 10.04 m, 8.58 s (3), 7.83 s (3), 4.77 s (2), 4.48 vw, 4.29 s (1), 3.90 s (2), 3.70 w, 3.45 w, 3.35 vw, 3.24 w, 3.05 vw, 2.81 vw, 2.71 w, 2.59 w, and 2.46 w.

Anal. Calc. for $C_{13}H_{21}NO_6S$: C, 48.87; H, 6.63; N, 4.38; S, 10.03. Found: C, 48.92; H, 6.42; N, 4.56; S, 9.98.

9-(2-Amino-2-deoxy-α,β-D-xylofuranosyl)adenine (**9a**). — Compound **1b** (10.08 g) and phosphorus pentasulfide[4] (7.7 g) in reagent-grade pyridine (350 ml) was stirred and heated for 2.5 h under reflux. The solution was cooled, diluted with 3 volumes of chloroform, and kept overnight at 5°. The mixture was filtered, and the filtrate was evaporated to a syrup which was dissolved in chloroform. The solution was washed successively with water, aqueous sodium hydrogen carbonate solution, and water, dried (magnesium sulfate), and evaporated to dryness, and the residue was further dried at 0.1 torr, to give 9.2 g of a yellow syrup (**2**).

Following the general procedure of Fox and co-workers[4], a solution of this syrup (9.2 g) in anhydrous methanol (15 ml) was added to anhydrous methanol (85 ml) nearly saturated at 0° with anhydrous ammonia. The mixture was sealed in a steel cylinder, heated for 18 h at 100°, cooled, and evaporated to dryness; water (50 ml) was added to the residue, and a small quantity of BioRad AG-1 X-2 (OH^-, 50–100 mesh) ion-exchange resin was added portionwise. The resin was filtered off and well washed with 7:3 methanol–water, and the filtrate and washings were combined, and evaporated to a syrup (**3**); yield 8.52 g.

This syrup (8.52 g) was dissolved in 100 ml of water containing 1.74 g of sodium hydrogen carbonate, and a solution of 3.86 g of 1-fluoro-2,4-dinitrobenzene in ethanol (100 ml) was added. The mixture was stirred for 30 min at room temperature, shaken overnight, and concentrated to a small volume. The residue was extracted with ethyl acetate, and the extract was washed with a cold (0°), saturated, aqueous solution of sodium hydrogen carbonate, and dried (magnesium sulfate). Evaporation of the solvent, and drying at 0.1 torr, gave crude, syrupy ethyl 2-deoxy-2-(2,4-dinitroanilino)-1-thio-α-D-xylofuranoside (**4**); yield 7.55 g.

This syrup (7.55 g) was acetylated with acetic anhydride (70 ml) and pyridine (70 ml), as described for the preparation of **1b**, and the product was isolated and dried in the same way. There resulted a yield of 8.95 g (63%, from **1b**) of syrupy ethyl 3,5-di-*O*-acetyl-2-deoxy-2-(2,4-dinitroanilino)-1-thio-α-D-xylofuranoside (**5**), which was homogeneous by t.l.c. with chloroform as the developer.

This acetylated material (8.95 g) was dissolved in 100 ml of dry dichloromethane, and the solution was treated for 10 min at 0° with dry chlorine, and evaporated to dryness. The residual syrup (**6**) was dried under vacuum (oil pump), and redissolved in dry dichloromethane (50 ml), and the solution was transferred to a hot, vigorously stirred, azeotropically dried suspension of *N*-acetyl(chloromercuri)adenine (15 g) and Celite (6 g) in toluene (250 ml). After removal of the dichloromethane by distillation, the mixture was refluxed (with protection from moisture) for 6 h, cooled, and filtered. The filter cake was well washed with warm chloroform and dichloromethane. The filtrate and washings were combined, and evaporated to dryness, and the residue was extracted with dichloromethane. The extract was washed successively with 30% aqueous potassium iodide, saturated aqueous sodium hydrogen carbonate, and water, dried (magnesium sulfate), and evaporated to a syrup; yield 6.07 g. T.l.c. on silica gel plates 200 × 200 × 1 mm), with ethyl acetate as the developer, gave a main zone of nucleoside material, R_F ~0.3, which was excised, and extracted with acetone. Evaporation of the solvent from the extract, and extraction of the residue with dichloromethane left, after final removal of solvent, a glass; yield 3.34 g (**7**, 30% from **2**). To a solution of this glass (3.34 g) in ethyl acetate (30 ml) and methanol (220 ml) was added 3.0 g of picric acid, and the solution was refluxed for 20 min. The crystalline picrate (**8**), formed on cooling, was filtered off, and washed with methanol; yield 3.43 g (77%), m.p. 160–168°.

Anal. Calc. for $C_{26}H_{23}N_{11}O_{16}$: N, 20.66. Found: 20.77.

To a stirred solution of the picrate (6.67 g) in acetone (480 ml) and water (120 ml) at 45–50° was added Bio Rad AG-1 X-2 (OH^-, 50–100 mesh) ion-exchange resin, portionwise, until the solution was decolorized. The resin was removed by filtration, and the filtrate was evaporated to dryness. The residue was triturated with dichloromethane until a filterable solid was obtained. The solid was filtered, and dissolved in aqueous methanol, and the solution was treated with activated carbon. The suspension was filtered, and the filtrate evaporated to dryness, whereupon crystallization occurred. The crystals of 9-(2-amino-2-deoxy-α,β-D-xylofuranosyl)-adenine (**9a**) were recrystallized from water–1-propanol; yield 866 mg (36%), m.p. 200–203°, $[\alpha]_D^{24}$ −17 ±3° (*c* 1.36, methanol); λ_{max}^{KBr} 2.90–3.10 (NH, OH), 6.06, 6.19, 6.37, and 6.78 μm (NH, purine) $\lambda_{max}^{H_2O}$ 262 nm (ε 14,400); n.m.r. spectral data (δ, D_2O): 3.85–4.98 (solvent and sugar-ring protons), 5.92 (0.5 proton, $J_{1',2'}$ 3.5 Hz, H-1'), 6.44 (0.5 proton, $J_{1',2'}$ 5.5 Hz, H-1'), 8.05, 8.22, 8.28 (2 protons, overlapping singlets, H-2', H-8'); X-ray powder diffraction data: 14.73 vw, 10.52 w, 7.69 w, 6.19 m, 5.50 w, 4.77 s (1), 3.76 s (2), 3.56 vw, 3.23 s (3), and 2.92 w.

Anal. Calc. for $C_{10}H_{14}N_6O_3$: C, 45.09; H, 5.30; N, 31.56. Found: C, 45.23; H, 5.67; N, 31.83.

T.l.c., with 1:1 ethyl acetate–methanol as the developer, revealed two spots of almost equal intensity, with R_F values of 0.24 and 0.31.

Separation of the anomeric D-*xylofuranosyl nucleosides* **9b** *and* **9c**. — The anomers were separated by the general procedure of Dekker[10]. A solution of the anomeric mixture **9a** (900 mg) in 30 ml of 9:1 water–methanol was siphoned onto a column (51 × 3.2 cm) of Bio Rad AG-1 X-2 (OH^-, 200–400 mesh) ion-exchange resin which had been pre-equilibrated with the same solvent mixture. Elution was begun with 9:1 water–methanol, 10-ml fractions being collected, with monitoring by a u.v. fraction-analyzer. At tube 68, the eluting solvent was changed to 4:1 water–methanol. At tubes 135–195, a u.v.-absorbing component issued from the column. At tube 202, the eluting solvent was changed to 3:7 water–methanol, and, at tube 235, a second u.v.-absorbing component began to issue from the column. At tube 244, the eluting solvent was changed to 1:1 water–ethanol. The second component was completely eluted at tube 295.

Tubes 135–195 (fraction 1) and tubes 235–295 (fraction 2) were separately pooled, and the solvent was removed from each by evaporation. The residue from fraction 1 was crystallized by dissolution in water, addition of propyl alcohol, and concentration. There resulted 305 mg (34%) of 9-(2-amino-2-deoxy-β-D-xylofuranosyl)adenine (**9c**), m.p. 187–189°, $[\alpha]_D^{24}$ −56 ±2° (*c* 1.00, methanol); the circular dichroism is recorded in Fig. 1; λ_{max}^{KBr} 2.90–3.10 (OH, NH), 5.91, 6.19, 6.39, and 6.76 μm (NH, purine); $\lambda_{max}^{H_2O}$ 260 nm (ε 14,900), $\lambda_{max}^{50\ mM\ HCl}$ 255 nm (ε15,300); n.m.r. data (δ D_2O): 3.85–4.98 (sugar-ring protons and solvent), 5.92 (1-proton doublet, $J_{1',2'}$ 3.5 Hz, H-1'), 8.05, 8.28 (2 protons, singlets, H-2, H-8); δ D_2O–pyridine-d_5 (1:1), 4.17 (H-2', $J_{2',3'}$ 2.5 Hz), 4.3–4.4 (H-5'), 4.6–4.9 (H-3' and H-4'), 6.21 (H-1', $J_{1',2'}$ 2.5 Hz), 8.37, 8.59 (H-2 and H-8); X-ray powder diffraction data: 9.82 m, 7.08 s (3), 6.46 s (1,1), 5.82 m, 5.53 s (1,1), 5.24 m, 4.87 s (3), 4.41 s (2), 4.17 m, 3.96 m, 3.81 m, 3.63 vw, 3.53 m, 3.38 w, 3.25 w, 3.06 w, 2.91 w, 2.82 vw, and 2.67 w.

Anal. Calc. for $C_{10}H_{14}N_6O_3$: C, 45.09; H, 5.30; N, 31.56. Found: C, 45.22; H, 5.39; N, 31.89.

This compound exhibited a single spot (R_F 0.24) on t.l.c. with 1:1 ethyl acetate–methanol as developer.

Fraction 2 was evaporated to dryness, and was obtained as a syrup (340 mg, 40%) that, on long standing, crystallized as a trihydrate. 9-(2-Amino-2-deoxy-α-D-xylofuranosyl)adenine trihydrate was obtained pure on two further recrystallizations from water, and was dried over potassium hydroxide at room temperature/4 torr; m.p. 138–139° (sintering at 76–77°), $[\alpha]_D^{22}$ +19 ±3° (*c* 1.03, methanol); the circular dichroism (anhydrous basis) is recorded in Fig. 1; X-ray powder diffraction data: 11.47 vw, 8.73 s (1), 7.36 m, 6.24 w, 5.65 m, 5.33 w, 4.88 m, 4.60 w, 4.37 s (2), 3.63 w, 3.47 s, 3.21 s (3), 3.11 m, 3.03 m, 2.95 s, 2.81 w, 2.75 w, and 2.53 m. The water of crystallization was readily removed by drying over phosphorus pentaoxide for 4 h at room temperature/10 mtorr.

Anal. Calc. for $C_{10}H_{14}N_6O_3 \cdot 3H_2O$: H_2O, 16.90. Found: 16.95 (loss in weight under above conditions).

The anhydrous form of 9-(2-amino-2-deoxy-α-D-xylofuranosyl)adenine (**9b**), obtained as described, was crystalline, m.p. 139–140°, $[\alpha]_D^{22}$ +23 ±3° (*c* 0.86, methanol, calculated from the rotation of the trihydrate); the circular dichroism (anhydrous basis) is recorded in Fig. 1; λ_{max}^{Nujol} 3.05, 3.10 (OH, NH), 6.05, 6.10, 6.24, and 6.35 μm (NH, purine); $\lambda_{max}^{H_2O}$ 258 nm (ε 15,700), $\lambda_{max}^{50\ mM\ HCl}$ 254 nm (ε 15,600); n.m.r. data [δ, D_2O–pyridine-d_5 (1:1)]: 4.20–4.35 (H-5′ and H-2′), 4.85–5.25 (H-3′ and H-4′), 6.76 (1-proton doublet, $J_{1',2'}$ 5.1 Hz, H-1′), 8.45, 8.58 (H-2 and H-8); X-ray powder diffraction data: 9.35 m, 8.26 s, 6.55 m, 5.56 m, 5.15 s (1), 4.69 s (3), 4.13 m, 3.75 w, 3.55 vw, 3.42 s (2), 3.26 s, 3.07 m, 2.75 w, and 2.54 m.

Anal. Calc. for $C_{10}H_{14}N_6O_3$: C, 45.09; H, 5.30; N, 31.56. Found: C, 44.98; H, 5.55; N, 31.54.

This compound gave one spot, R_F 0.31, on t.l.c. with 1:1 ethyl acetate–methanol as the developer.

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REACTION OF ACETATES OF NEOHESPERIDOSE, RUTINOSE, AND SOME FLAVONOID L-RHAMNOSYL-D-GLUCOSIDES WITH DIHALOMETHYL METHYL ETHERS

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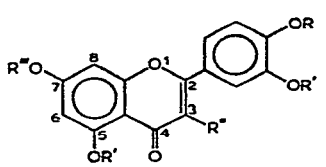
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ABSTRACT

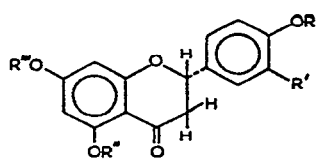
Conversion of the β-hepta-acetate into the *O*-acetylglycosyl halide by means of dihalomethyl methyl ethers occurs more rapidly with rutinose (6-*O*-α-L-rhamnopyranosyl-D-glucose) than with neohesperidose (2-*O*-α-L-rhamnopyranosyl-D-glucose). Further cleavage of the glycosidic link between the L-rhamnose and D-glucose residues occurs readily with dibromomethyl methyl ether, but to a very much lesser extent with the dichloro analogue. Dihalomethyl methyl ethers liberate oligosaccharides less selectively from flavonoid 7-(L-rhamnosyl-D-glucosides) than from the less acid-resistant 3-glycoside, rutin. A method is described for the preparation of β-rutinose hepta-acetate in good yield from rutin deca-acetate.

INTRODUCTION

Recently, the use was reported of dihalomethyl methyl ethers for the selective cleavage of *O*-acetyloligosaccharide glycosides to yield *O*-acetyloligosaccharyl halides[1]. Although various methods have been employed for the selective cleavage of glycosidic linkages, the high yields of acetylated rutinosyl halides obtained from rutin deca-acetate (**1**) by the new procedure suggested that it might be of particular value as a general method for preparing and identifying oligosaccharides from flavonoid compounds. The present investigation was undertaken with a view to extending the reaction to other flavonoid L-rhamnosyl-D-glucosides after establishing the stability of the disaccharide acetates under various conditions.



1 R=R′=R‴=Ac; R″=Hexa-*O*-acetyl-β-rutinosyloxy
2 R=Me; R′=Ac; R″=H; R‴=Hexa-*O*-acetyl-β-rutinosyl
3 R=R′=R‴=Ac; R″=OAc
4 R=R′=R‴=Me; R″=OMe



5 R=Me; R′=OAc; R″=Ac; R‴=Hexa-*O*-acetyl-β-rutinosyl
6 R=R‴=Ac; R′=H; R″=Hexa-*O*-acetyl-β-neohesperidosyl

TABLE I

REACTION OF NEOHESPERIDOSE, RUTINOSE, AND RUTIN ACETATES (0.04 MMOLE) WITH DIBROMOMETHYL METHYL ETHER (0.05 ML) IN CHLOROFORM (0.6 ML) IN THE PRESENCE OF ZINC CHLORIDE (20 MG)

Expt.	*Starting material*	*Bromide formed*	*Time (min)*			
			2	*5*	*30*	*60*
1	β-Neohesperidose hepta-acetate		+++	trace	—	—
		Hexa-*O*-acetyl-α-neohesperidosyl	++	++++	+	—
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	trace	+++	++++
2	β-Rutinose hepta-acetate		—	—	—	—
		Hexa-*O*-acetyl-α-rutinosyl	++++	++++	—	—
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	trace	++++	++++
3	β-Neohesperidose hepta-acetate		++++	++++	++++	++++
	[in presence of 0.04 mmole of	Hexa-*O*-acetyl-α-neohesperidosyl	—	—	+	+
	penta-*O*-acetylquercetin (3)]	Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	—	—	—
4	β-Neohesperidose hepta-acetate		++++	++++	++	trace
	[in presence of 0.04 mmole of	Hexa-*O*-acetyl-α-neohesperidosyl	—	trace	++	++++
	penta-*O*-methylquercetin (4)]	Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	—	trace	+
Control	Rutin deca-acetate (1)	Hexa-*O*-acetyl-α-rutinosyl	trace	+	++	+++
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	—	trace	+

EXPERIMENTAL AND RESULTS

General. — M.ps. are uncorrected, and were determined by the Kofler method. Merck Kieselgel G was employed for thin-layer chromatography (t.l.c.), with benzene–acetone (9:1) as irrigant. Spot intensities were estimated visually after spraying with sulphuric acid, and heating for 15 min at 140°.

Preparation of reagents and samples. — The procedure of Gross and Karsch[2] was employed for the preparation of dibromomethyl methyl ether, and dichloromethyl methyl ether was prepared by the method of Rieche, Gross, and Höft[3].

Hesperidin, naringin, quercetin, and rutin (Koch–Light Laboratories) were acetylated with acetic anhydride–pyridine, and recovered from water in the usual way. Diosmin octa-acetate (**2**) was prepared from hesperidin octa-acetate (**5**) by dehydrogenation with selenium dioxide in acetic anhydride[4]. Penta-*O*-methylquercetin (**4**) was prepared by treatment of quercetin with methyl sulphate and potassium carbonate in acetone[5]. β-Neohesperidose hepta-acetate was synthesized as described by Koeppen[6], and β-rutinose hepta-acetate was prepared from compound **1** as described below. All samples were dried to constant weight at 110° under diminished pressure before use.

Reactions with dibromomethyl methyl ether. — A solution of the compound (0.04 mmole) and zinc chloride (20 mg) in chloroform (0.6 ml) was boiled, and dibromomethyl methyl ether (0.05 ml) was added. The reaction was monitored by t.l.c. A control with compound **1** was run in each case, and the compounds were studied both individually and simultaneously. Tables I and II contain the results of several experiments in which no differences in the reaction rates of the rutin acetate controls could be detected by t.l.c.

Reaction with dichloromethyl methyl ether. — Solutions of neohesperidose and rutinose acetates (0.04 mmole) in chloroform (0.6 ml), with various quantities of zinc chloride, were boiled, and examined periodically by t.l.c. after addition of various amounts of dichloromethyl methyl ether (Table III). Flavonoid L-rhamnosyl-D-glucoside acetates were examined as follows. A solution of the compound (0.04 mmole) and zinc chloride (40 mg) in chloroform (0.6 ml) was boiled, and treated with dichloromethyl methyl ether (0.1 ml). A control with compound **1** was run in each case, and the reaction mixtures were examined by t.l.c. after 1, 3, and 6 h (Table IV).

*Preparation of 1,2,3,4-tetra-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-6-deoxy-α-*L*-manno-pyranosyl)-β-*D*-glucopyranose (β-rutinose hepta-acetate, cf. ref. 1).* — A mixture of rutin deca-acetate (**1**; 1.0 g), dry, finely powdered zinc chloride (1.0 g, analytical reagent grade), and anhydrous chloroform (15 ml) was boiled, and dichloromethyl methyl ether (2.5 ml) was added. After 3 h, the solution was decanted, and the residue was washed with chloroform (2 × 10 ml). The combined chloroform solutions were washed with iced water, saturated, aqueous sodium hydrogen carbonate, and water until the washings were neutral to litmus, filtered through anhydrous sodium sulphate, and evaporated to dryness under diminished pressure at 30°. The dry residue (0.78 g)

TABLE II

REACTION OF FLAVONOID L-RHAMNOSYL-D-GLUCOSIDE ACETATES (0.04 MMOLE) WITH DIBROMOMETHYL METHYL ETHER (0.05 ML) IN CHLOROFORM (0.6 ML) IN THE PRESENCE OF ZINC CHLORIDE (20 MG)

Expt.	*Starting material*	*Bromide formed*	*Time (min)*		
			15	*60*	*120*
1	Diosmin octa-acetate (2)	Hexa-*O*-acetyl-α-rutinosyl	—	—	—
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	+	+++	++++
2	Hesperidin octa-acetate (5)	Hexa-*O*-acetyl-α-rutinosyl	+	+	++
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	trace	++	+++
3	Naringin octa-acetate (6)	Hexa-*O*-acetyl-α-neohesperidosyl	—	trace	++
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	trace	+	++
Control	Rutin deca-acetate (1)	Hexa-*O*-acetyl-α-rutinosyl	++	+++	++++
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	trace	+	+

TABLE III

REACTION OF NEOHESPERIDOSE AND RUTINOSE ACETATES (0.04 MMOLE) IN CHLOROFORM (0.6 ML) WITH DICHLOROMETHYL METHYL ETHER UNDER VARIOUS CONDITIONS

Dichloromethyl methyl ether (ml)	*Zinc chloride (mg)*	*β-Hepta-acetate*	*Time required for complete conversion into α-chloride (h)*	*Tri-O-acetyl-α-L-rhamnopyranosyl chloride detected after 6 h*
0.05	20	Neohesperidose	5	trace
0.05	20	Rutinose	1	+
0.10	20	Neohesperidose	4.25	trace
0.05	0	Neohesperidose	No reaction after 6 h	0
0.05	40	Neohesperidose	5	trace
0.10	40	Neohesperidose	2	+
0.10	40	Rutinose	0.25	++

was treated with a solution of mercuric acetate (0.8 g) in glacial acetic acid (16 ml) for 2 h at room temperature, the mixture was diluted with chloroform (50 ml), washed with water (5 × 20 ml), and evaporated to dryness, and the residue was dissolved in boiling ethanol (25 ml). A yellow precipitate (0.08 g) which formed on cooling was removed by filtration and discarded. On further standing, the filtrate deposited colourless prisms of β-rutinose hepta-acetate (0.261 g, 43.3%), m.p. 165–167°. The mother liquor was purified by t.l.c., the major component being located on the chromatoplates by spraying with water. After elution with acetone, and crystallization from ethanol, a further amount (0.098 g, 16.0%) of β-rutinose hepta-acetate, m.p. 168–169°, was obtained; total yield, 0.359 g (59.3%). After one recrystallization from ethanol, the pure compound had m.p. 169°, $[\alpha]_D^{19}$ −30.5° (*c* 2.56, chloroform); lit.[7], m.p. 168–169°, $[\alpha]_D^{24}$ −29.7° (chloroform).

β-Rutinose hepta-acetate was similarly prepared from compound **1** (1.0 g) in chloroform (15 ml) by treatment with zinc chloride (0.5 g) and dibromomethyl methyl ether (1.2 ml); a reaction period of 2 h generally gave the best results. However, as the highest yield of pure compound was only 32% by this procedure, the method employing dichloromethyl methyl ether is to be preferred.

DISCUSSION

The results presented in Table I reveal a significant difference in the rate of reaction of dibromomethyl methyl ether with the β-hepta-acetates of rutinose and neohesperidose. Whereas the former was completely converted into the α-bromide within 2 min, less than 50% of the latter had undergone conversion during this period, and a trace of unreacted acetate was still apparent after 5 min. Under similar conditions, but with the less-reactive dichloromethyl methyl ether, this difference in conversion rates was very much greater (Table III). Thus, conversion of β-rutinose hepta-acetate into the α-chloride was complete after 1 h, whereas conversion of β-neohesperidose hepta-acetate required a reaction period of 5 h.

Despite the high yields of *O*-acetylrutinosyl halides obtained from compound **1** by previous authors[1], the present results show that attack of the dihalomethyl methyl ethers at the link between the aglycone group and the disaccharide is by no means specific, nor is it in some cases even preferential (Table II). Both rutinose and neohesperidose acetates, after conversion into the α-bromides, undergo further attack at the glycosidic bond between the L-rhamnose and D-glucose residues, resulting in complete cleavage within 30 and 60 min respectively (Table I). The apparent, greater stability of this linkage in hexa-*O*-acetyl-α-rutinosyl bromide, formed from rutin acetate (**1**), indicated a protective effect due to the quercetin moiety. This was clearly demonstrated with β-neohesperidose hepta-acetate when the reaction was studied in the presence of equivalent amounts of penta-*O*-acetyl- (**3**) or penta-*O*-methyl-quercetin (**4**) (Table I). T.l.c. revealed that dibromomethyl methyl ether reacted preferentially with acetate **3**, converting it rapidly and quantitatively into a more

TABLE IV

REACTION OF FLAVONOID L-RHAMNOSYL-D-GLUCOSIDE ACETATES (0.04 MMOLE) WITH DICHLOROMETHYL METHYL ETHER (0.1 ML) IN CHLOROFORM (0.6 ML) IN THE PRESENCE OF ZINC CHLORIDE (40 MG)

Expt.	*Starting material*	*Chloride formed*	*Time (h)*		
			1	*3*	*6*
1	Diosmin octa-acetate (2)	Hexa-*O*-acetyl-α-rutinosyl	—	+	trace
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	trace	++	++
2	Hesperidin octa-acetate (5)	Hexa-*O*-acetyl-α-rutinosyl	++	++	++
		Tri-*O*-acetyl-α-L-rhamnopyranosly	—	trace	trace
3	Naringin octa-acetate (6)	Hexa-*O*-acetyl-α-neohesperidosyl	trace	+	+
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	trace	trace
Control	Rutin deca-acetate (1)	Hexa-*O*-acetyl-α-rutinosyl	+++	++++	++++
		Tri-*O*-acetyl-α-L-rhamnopyranosyl	—	trace	+

mobile product. Consequently, the neohesperidose acetate remained largely unchanged, even after 60 min. With ether **4**, conditions were established which more closely paralleled the rate of formation of *O*-acetylrutinosyl bromide from **1**; conversion of β-neohesperidose hepta-acetate into the α-bromide required an extension of reaction time from 5 to 60 min, and, as with **1**, only a small proportion of *O*-acetyl-L-rhamnosyl bromide was formed.

Substitution of dichloromethyl methyl ether for the dibromo analogue resulted in a marked, general decrease in reactivity, and no significant cleavage of disaccharide to monosaccharide was observed, even after 6 h. Increasing the concentration of the ether or zinc chloride alone had no significant effect on the rate of conversion of β-neohesperidose hepta-acetate into the α-chloride, although the catalytic effect of zinc chloride is clearly illustrated (Table III). However, doubling the concentration of both the ether and zinc chloride decreased the required reaction time by about 75% without causing any significant increase in the formation of *O*-acetyl-L-rhamnosyl chloride, except after 6 h. These modified conditions were subsequently adopted in experiments with flavonoid L-rhamnosyl-D-glucoside acetates (Table IV), as the rate of formation of *O*-acetylrutinosyl halide from **1** was similar to that observed under the experimental conditions with dibromomethyl methyl ether (Table II).

From Tables II and IV, it would appear that the use of dihalomethyl methyl ethers for the selective cleavage of oligosaccharides from flavonoid compounds is of limited applicability. This is not altogether unexpected if resistance to acid hydrolysis is taken as an index of the general stability of glycosidic bonds. Flavonol 3-glycosides, for instance, are cleaved under fairly mild conditions of acid hydrolysis, and the use of boiling, 10%, aqueous acetic acid has been described[8] as a preparative procedure for obtaining rutinose from rutin, indicating that the glycosidic link between L-rhamnose and D-glucose is more stable than that between D-glucose and quercetin in this compound. Many 7-glycosyloxyflavonoids, on the other hand, are hydrolyzed only under quite drastic conditions, and at 100°, hydrolysis of diosmin occurs only slowly, even in concentrated sulphuric acid[9]. Similar findings were obtained in the present study of the action of dihalomethyl methyl ethers on flavonoid 7-(L-rhamnosyl-D-glucosides). With both the dichloro- and dibromo-methyl methyl ethers, the tri-*O*-acetyl-α-L-rhamnopyranosyl halide was the main cleavage product from diosmin acetate (**2**), and insufficient disaccharide was liberated from hesperidin and naringin acetates (**5**, **6**) for adaptation on a preparative scale to be of much value.

With **1**, however, the findings of Bognár *et al.*[1] were confirmed. Cleavage of the oligosaccharide is best accomplished with dichloromethyl methyl ether, although, even with the dibromo analogue, β-rutinose hepta-acetate can be prepared in higher yield (32%) than by the acetic acid method[8] of Zemplén and Gerecs (net yield, 22%). In the present modified procedure, this compound was prepared directly from the intermediate *O*-acetyl-α-rutinosyl halide (not isolated) by reaction with mercuric acetate in acetic acid. It is of interest that mercuric acetate in benzene catalyzes the condensation of 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide with 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl chloride to yield hexa-*O*-acetyl-α-rutinosyl chloride[7],

whereas, in acetic acid, it converts this product quantitatively at room temperature into the β-hepta-acetate.

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CHARACTERISATION OF 2-AMINO-2-DEOXY-D-GLUCOSE, 2-AMINO-2-DEOXY-D-GALACTOSE, AND RELATED COMPOUNDS, AS THEIR TRIMETHYLSILYL DERIVATIVES BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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ABSTRACT

Combined gas–liquid chromatography–mass spectrometry (GC-MS) has been used for the analysis of 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, their methyl glycosides, and the corresponding alditols, as well as of the *N*-acetylated forms. The compounds were analyzed as trimethylsilyl (TMS) derivatives synthesized by using chlorotrimethylsilane and hexamethyldisilazane in pyridine, or using these reagents together with bis(trimethylsilyl)acetamide. In the latter procedure, the free amino groups, in addition to the hydroxyl groups, are also trimethylsilylated. Identification of the derivatives was based on their retention times in gas–liquid chromatography and their mass-spectral characteristics. The value of GC-MS of TMS derivatives in the analysis of polysaccharides is discussed.

INTRODUCTION

Trimethylsilyl (TMS) derivatives of carbohydrates have found wide application in the quantitative analysis of monosaccharides by gas–liquid chromatography[1,2]. The possibility of recovering the original carbohydrate from its TMS derivative, as well as the suitability of TMS ethers of mono- and oligo-saccharides for analysis by mass spectrometry[3–5], makes these derivatives particularly interesting from the point of view of the structural analysis of polysaccharides.

Carbohydrates of animal origin, *e.g.*, those occurring in glycoproteins, mucopolysaccharides, and glycolipids, contain not only simple hexoses but also *N*-acetylated amino sugars. The commonest of these amino sugars are glucosamine (2-amino-2-deoxy-D-glucose) and galactosamine (2-amino-2-deoxy-D-galactose). The conditions required for their quantitative liberation by hydrolysis or methanolysis cause cleavage of *N*-acetyl groups, in addition to the rupture of glycosidic bonds. Even the milder conditions used for production of oligosaccharides by partial hydrolysis bring about varying degrees of *N*-deacetylation.

Both the amino sugars and their *N*-acetylated derivatives can be quantitatively analyzed as TMS derivatives[6–8]. Our preliminary studies on the galactosamine TMS derivative synthesized from the hydrochloride by treatment with chloro-

trimethylsilane and hexamethyldisilazane in pyridine showed that, in the former case, the amino groups are not silylated[9].

The purpose of the present work was to investigate the usefulness of gas–liquid chromatography–mass spectrometry (GC-MS) of TMS derivatives in the structural analysis of polysaccharides for the identification of amino sugars and various derivatives thereof. A TMS derivative in which all of the functional groups were trimethylsilylated was synthesized by use of bis(trimethylsilyl)acetamide[10] (BSA). The possibility of employing these derivatives in quantitative and qualitative analysis was also investigated.

RESULTS

The relative retention times of TMS derivatives of glucosamine, galactosamine, the *N*-acetyl derivatives, methyl glycosides, and the corresponding alditols in g.l.c. on SE-30 and QF-1 liquid phases are presented in Table I. The TMS derivatives were synthesized by using an excess of chlorotrimethylsilane–hexamethyldisilazane–pyridine (0.5:1:10) for 30 min at room temperature. Analysis of the derivatives by GC-MS (see below) showed that only the hydroxyl groups had undergone trimethylsilylation. However, small proportions of *N*-trimethylsilylated derivatives were found if the reaction was carried out at 70° or if the concentration of the silanes in the silylation mixture was increased. Of the two stationary phases used, QF-1 was more suitable for separation of anomers but less useful for separating galactosamine derivatives from glucosamine derivatives than was SE-30. On the latter liquid phase, glucosamine, its methyl glycoside, the *N*-acetylated derivatives and the *N*-acetylated alditol were separated from the corresponding galactosamine derivatives.

TABLE I

RELATIVE RETENTION TIMES OF TMS DERIVATIVES OF AMINO SUGARS AND RELATED COMPOUNDS SYNTHESIZED WITHOUT BSA

Compound	*Stationary phase*		
	SE-30 (187°)[a]	*QF-1 (140°)*[a]	*QF-1 (200°)*[b]
2-Amino-2-deoxy-D-glucose	0.88 1.12	1.20 1.38	—
2-Amino-2-deoxy-D-galactose	0.77	1.11 1.22	—
Methyl 2-amino-2-deoxy-D-glucoside	0.70	1.02	—
Methyl 2-amino-2-deoxy-D-galactoside	0.59	1.06	—
2-Acetamido-2-deoxy-D-glucose	2.25	8.3 10.5	0.55 0.70
2-Acetamido-2-deoxy-D-galactose	2.04	—	0.55 0.70
Methyl 2-acetamido-2-deoxy-D-glucoside	2.05	—	0.70
Methyl 2-acetamido-2-deoxy-D-galactoside	1.77	—	0.67
2-Amino-2-deoxy-D-glucitol	1.15	1.44	—
2-Amino-2-deoxy-D-galactitol	1.15	1.67	—
2-Acetamido-2-deoxy-D-glucitol	2.12	—	0.38
2-Acetamido-2-deoxy-D-galactitol	2.23	—	0.45

[a]Retention times relative to α-D-glucose. [b]Retention times relative to α-D-lactose.

Penta-*O*-TMS-aminodeoxyhexitols were not separated on this liquid phase but could be resolved on QF-1. The presence of an *N*-acetyl group in a molecule increased the retention time of a monosaccharide on the QF-1 liquid phase approximately 10-fold. The corresponding effect on the SE-30 liquid phase was small.

Silylation with bis(trimethylsilyl)acetamide (BSA). Attempts to trimethylsilylate glucosamine with only BSA in pyridine were not successful, because of the appearance of multiple g.l.c. peaks even after heating at 70° for several hours; the main component was the penta-trimethylsilyl derivative. Better results were achieved when BSA was used together with the other silylation reagents. Conversion into the completely silylated derivative could be effected with BSA–chlorotrimethylsilane–hexamethyldisilazane–pyridine (1:0.5:1:10) within 30 min at room temperature. These derivatives were present as one or two symmetrical peaks on SE-30 and had longer retention times than the corresponding compounds with a free amino group (Table II). The anomers of the penta-trimethylsilyl derivatives of both glucosamine and galactosamine were separated on SE-30, but partial overlapping of the galactosamine peaks with those of glucosamine occurred.

TABLE II

RELATIVE RETENTION TIMES OF TMS DERIVATIVES OF AMINO SUGARS AND RELATED COMPOUNDS SYNTHESIZED WITH BSA

Compound	*Stationary phase SE-30 (187°)*[a]	
2-Amino-2-deoxy-D-glucose	1.10	1.32
2-Amino-2-deoxy-D-galactose	0.97	1.32
Methyl 2-amino-2-deoxy-D-glucoside	1.00	
Methyl 2-amino-2-deoxy-D-galactoside	0.82	
2-Amino-2-deoxy-D-glucitol	1.53	
2-Amino-2-deoxy-D-galactitol	1.49	

[a]Retention times relative to α-D-glucose.

The Si–N bond was found to be very labile as compared to the Si–O bonds, for the chromatograms often revealed the presence of varying amounts of the corresponding TMS derivative having a free amino group. Although this is probably caused, in part, by incomplete silylation initially, some decomposition undoubtedly occurs during g.l.c. Extraction into hexane likewise caused hydrolysis of the Si–N bond. After standing in a hexane solution for 1–2 h at room temperature, most of the penta-TMS-glucosamine was converted into a tetra-*O*-TMS derivative, which could easily be resilylated to a penta-TMS derivative with BSA. Decomposition was considerably less when the injections into the gas chromatograph were made directly from the silylation mixture.

Mass spectrometry. All of the TMS derivatives mentioned in Tables I and II were subjected to analysis with GC-MS. As with other sugar derivatives previously studied[11,12], differences between the mass spectra of anomeric and other stereo-

isomeric forms were very small, and no attempts were made to use the spectra for differential analysis of isomers. Mass spectra of the different types of TMS derivative of amino sugars are presented in Figs. 1 and 2. For comparison, spectra of TMS derivatives of D-glucose, methyl D-mannopyranoside, and D-mannitol are also included. In general, the fragmentation of TMS derivatives of reducing sugars or their methyl glycosides seems to follow uniform principles, whereas alditols exhibit

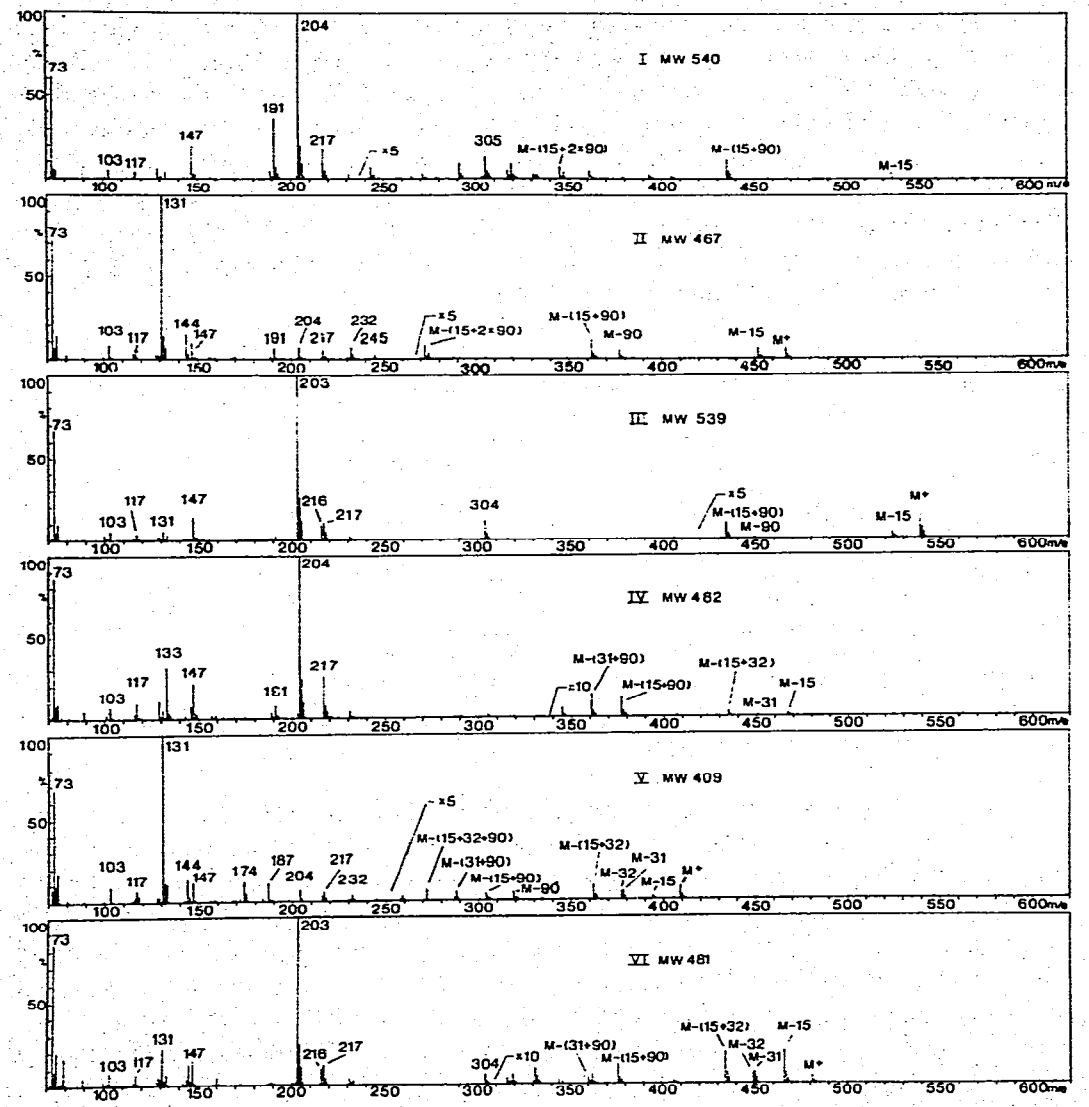


Fig. 1. Mass spectra of penta-*O*-(trimethylsilyl)-β-D-glucose (I), 2-amino-2-deoxy-tetra-*O*-(trimethylsilyl)-D-galactose (II), 2-deoxy-tetra-*O*-(trimethylsilyl)-2-(trimethylsilylamino)-D-galactose (III), methyl tetra-*O*-(trimethylsilyl)-α-D-mannopyranoside (IV), methyl 2-amino-2-deoxy-tri-*O*-(trimethylsilyl)-D-galactoside (V), and methyl 2-deoxy-tri-*O*-(trimethylsilyl)-2-(trimethylsilylamino)-D-galactoside (VI). Stationary phase in g.l.c., SE-30.

different fragmentation characteristics. Because isotope labelling was not used in these studies, the interpretation of the fragmentation patterns should be considered tentative.

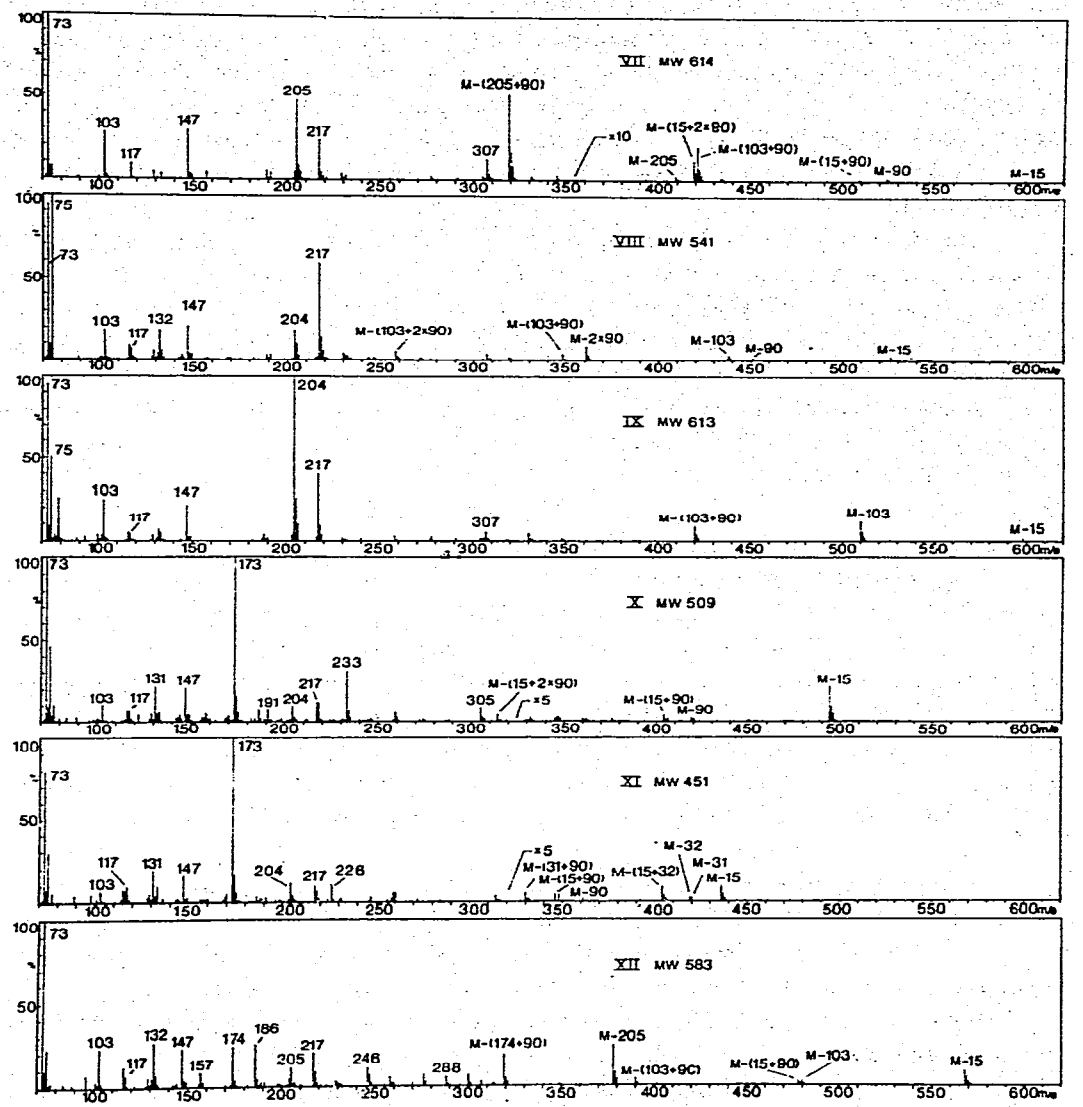


Fig. 2. Mass spectra of hexa-*O*-(trimethylsilyl)-D-mannitol (VII), 2-amino-2-deoxy-penta-*O*-(trimethylsilyl)-D-glucitol (VIII), 2-deoxy-penta-*O*-(trimethylsilyl)-2-(trimethylsilylamino)-D-glucitol (IX), 2-acetamido-2-deoxy-tetra-*O*-(trimethylsilyl)-D-galactose (X), methyl 2-acetamido-2-deoxy-tri-*O*-(trimethylsilyl)-D-glucoside (XI), and 2-acetamido-2-deoxy-penta-*O*-(trimethylsilyl)-D-galactitol (XII). Stationary phase in g.l.c. SE-30.

The ions at *m/e* 73, 103, 117, and 147, common to a number of TMS derivatives[13,14] were present in all of the spectra, although their relative intensity varied.

The molecular ion was frequently observed in the mass spectra of the TMS derivatives of the aminodeoxyhexoses and their methyl glycosides, but was absent in the spectra of the other compounds studied. The ion M−15 was always present, and it can be used together with ions M−90, M−(15+90), and M−(15+2×90) for determinations of molecular weight. It is evident that these ions are formed from the parent molecule through losses of the methyl and trimethylsilanol moieties. TMS derivatives of methyl glycosides are characterized in the high-mass range by the presence of the ions M−15, M−31, M−32, M−(15+32), M−(31+90), and M−(15+32+90), which probably arise from sequential losses of methyl, methoxyl, methanol, and trimethylsilanol moieties.

The base peak in the mass spectra of all TMS derivatives possessing a hemiacetal ring can be attributed to an ion $(TMS\text{-}O\text{-}CH{=}CH\text{-}R)^+$, where R can be O-TMS (*m/e* 204), NH-TMS (*m/e* 203), $NH\text{-}CO\text{-}CH_3$ (*m/e* 173), or NH_2 (*m/e* 131). The peak at *m/e* 217, corresponding to the ion $TMS\text{-}O\text{-}CH{=}CH\text{-}CH{=}O^+\text{-}TMS$, was present in the spectra of all of the TMS derivatives examined. The spectra of tetra-*O*-TMS derivatives of hexosamines and tri-*O*-TMS derivatives of their methyl glycosides showed a peak of still greater intensity at *m/e* 144, which can be explained by the formation of an analogous fragment in which one *O*-TMS group is replaced by NH_2. Similarly, the corresponding derivatives containing an NH-TMS group showed a fragment at *m/e* 216.

The occurrence of the relatively intense peak at *m/e* 305 in the spectrum of the TMS derivative of D-glucose can be rationalized by assigning to it a structure $TMS\text{-}O\text{-}CH{=}C(O\text{-}TMS)\text{-}CH{=}O^+\text{-}TMS$ analogously with methyl ethers[5]. Substitution of an O-TMS group by NH_2 or NH-TMS, as in the tetra-*O*-TMS and penta-TMS derivatives of aminodeoxyhexoses and the corresponding methyl glycosides, leads to the ions at *m/e* 232 and *m/e* 304, respectively. The intense peak at *m/e* 174 in the spectra of tri-*O*-TMS derivatives of methyl 2-amino-2-deoxyhexosides can be explained by the substitution of two of the O-TMS groups by $O\text{-}CH_3$ and NH_2 in this ion.

Aminodeoxyhexoses having an unsilylated amino group exhibit a relatively intense peak at *m/e* 245. This is accompanied by a peak at *m/e* 187 (245−58) in the spectra of the corresponding methyl glycosides, which suggests that these peaks are produced by ions containing the amino group and the functional group at C-1.

Most of the fragments in the spectra of trimethylsilylated alditols, apart from those formed by elimination of the methyl and trimethylsilanol groups from the parent molecule, arise by direct cleavage of C–C bonds in the carbon chain, often accompanied by expulsion of trimethylsilanol. Elimination of C-1 with its substituents leads to ions at *m/e* M−103, M−(103+90), and M−(103+2×90). Cleavage of the C-2–C-3 bond produces ions at *m/e* 205, 409 (M−205), and 319 (M−205−90) from the TMS derivative of D-mannitol. Although the same three fragments can also be formed from the TMS derivatives of other alditols, the presence of the amino group changes the charge distribution. This is clearly seen in the mass spectrum of the hexa-TMS derivative of 2-amino-2-deoxy-D-galactitol, where the ion at

m/e 204 forms the base peak. The spectra of TMS derivatives of 2-acetamido-2-deoxyhexitols are somewhat more complicated than the others, most probably because of the presence of ions formed by elimination of acetic acid (60) or ketene (42) from the primary fragments.

DISCUSSION

Analysis by GC-MS showed that the ordinary procedure for the trimethylsilylation of sugars with chlorotrimethylsilane and hexamethyldisilazane in pyridine is not sufficient for the silylation of the free amino group of 2-amino-2-deoxyhexose derivatives. Reaction of all functional groups can be effected, as already suggested by Sweeley[9], by adding BSA to the reaction mixture. However, completely silylated derivatives appear to be less suitable for quantitative determinations, owing to the lability of the Si-N bond and also because of overlapping of the glucosamine and galactosamine peaks in g.l.c. As *N*-trimethylsilylation can be accomplished with BSA, also using an amino sugar trimethylsilyl ether as substrate, the procedure may possibly be of value for tentative identifications of amino groups in sugars by g.l.c.

Examination of the mass spectra of the different TMS derivatives has shown that mass spectrometry can be used for identifying a compound as a hexosamine (or the derived alditol) and determining the number and nature of the possible substituents. Although differentiation between glucosamine and galactosamine derivatives is not possible on the basis of their mass spectra, these two stereoisomers can be separated by g.l.c. on SE-30 or QF-1 liquid phases. Combined GC-MS therefore offers a valuable method for the analysis of polysaccharides, which contain amino sugars, from biological sources. In view of the structural analysis of glycolipids, glycoproteins, and mucopolysaccharides by partial hydrolysis, the mass spectra of *N*-acetylated amino sugars are of special importance, because most of the fragments are to be found in the mass spectra of oligosaccharides containing *N*-acetylated amino sugars.

EXPERIMENTAL

D-Glucosamine hydrochloride, D-galactosamine hydrochloride, and their *N*-acetyl derivatives were purchased from Mann Research Laboratories, Inc., New York, N. Y. D-Glucose and D-mannose were bought from B.D.H. Ltd., Poole, Great Britain. All reducing sugars were equilibrated in water for a minimum of two days before use.

Methyl glycosides of amino sugars and their *N*-acetyl derivatives were synthesized from the corresponding acetamidodeoxyhexoses by treatment with 0.5N dry, methanolic sulphuric acid at 70° for 18 h and 2 h, respectively, followed by removal of sulphate with Dowex-1 (acetate form). Amino sugar methyl glycosides were further purified with Dowex-50 (H^+). Methyl D-mannopyranoside was prepared from D-mannose, using 0.5N methanolic sulphuric acid for 18 h at 70°. 2-Acetamido-2-deoxyhexitols and D-mannitol were prepared by reduction, with potassium boro-

hydride, of the corresponding reducing sugars. 2-Acetamido-2-deoxyhexitols were deacetylated with N sulphuric acid for 3 h at 100°, followed by purification of the aminodeoxyhexitols with Dowex-50 (H^+).

Chlorotrimethylsilane (Fluka A.G.) and hexamethyldisilazane (Fluka A.G.) were distilled before use. Pyridine was distilled from KOH and stored over KOH pellets. Bis(trimethylsilyl)acetamide (Pierce Chemical Company, Rockford, Ill.) was used as supplied.

A Perkin–Elmer Model 900 gas chromatograph, equipped with a hydrogen flame-ionization detector, was used in the g.l.c. experiments. Coiled glass columns (2 m × 3.5 mm), filled with packings of 3% QF-1 on Gas Chrom Q (80–100 mesh) or 2.2% SE-30 on Gas Chrom S (100–120 mesh), were used (support and phases from Applied Science Laboratories, Inc., State College, Pa., U. S. A.). Column temperature: QF-1, 140° and 200°; SE-30, 187°. The temperatures of the flash heaters and detectors were about 60° higher than the column temperatures. Argon was used as carrier gas, with a flow of about 15 ml/min.

Gas–liquid chromatography–mass spectrometry was performed with an LKB Model 9000 Gas Chromatograph–Mass Spectrometer (LKB-Produkter Ab, Stockholm, Sweden), using QF-1 and SE-30 columns with helium as carrier gas. Column conditions were as in conventional g.l.c.; the temperatures of the molecule separator and the ion source were 240° and 290°, respectively. The energy of the bombarding electrons was 70 eV; the ionizing current was 60 μamps. Mass spectra were taken with a scan speed of 4–6 seconds (*m/e* 20–650).

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APIOSE. II.* 1,2:3,5-Di-*O*-ISOPROPYLIDENE-α-D-APIO-D-FURANOSE AND STEREOSELECTIVE SYNTHESES OF 3-DEOXY-1,2-*O*-ISOPROPYLIDENE-α-D-APIO-L-FURANOSE AND 3,5-ANHYDRO-1,2-*O*-ISOPROPYLIDENE-α-D-APIO-L-FURANOSE

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ABSTRACT

Isolation of the second isomer of di-*O*-isopropylidene-D-apiose, namely, 1,2:3,3[1]-di-*O*-isopropylidene-[3-*C*-(hydroxymethyl)-α-D-erythrofuranose] (**2**), has been achieved. Graded, acid hydrolysis gave the corresponding 1,2-*O*-isopropylidene derivative (**3**). High stereoselectivity was observed in the hydroboration of 3-deoxy-1,2-*O*-isopropylidene-3-*C*-methylene-D-*glycero*-tetrose (**4**), and acid hydrolysis of the product afforded 3-deoxy-D-apiose, which may be differentiated from 3-deoxy-D-*erythro*-pentose by its color reaction with *p*-anisidine hydrochloride. Epoxidation of **4** with *m*-chloroperoxybenzoic acid was also highly stereoselective, and gave a *spiro*-epoxide. A synthesis of the same epoxide (**8**) from 1,2-*O*-isopropylidene-5-*O*-*p*-tolylsulfonyl-α-D-apio-L-furanose (**7**) established that epoxidation occurred *trans* to the isopropylidene ring, as anticipated. The minor product (4%) of the epoxidation reaction was shown to be identical chromatographically with 3,5-anhydro-1,2-*O*-isopropylidene-α-D-apio-D-furanose (**9**), which was prepared by the reaction of 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**10**) with dimethylsulfonium methylide.

INTRODUCTION

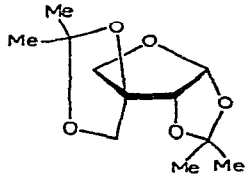
In Part I of this series[1], the structure of the known diisopropylidene acetal of D-apiose was established as 1,2:3,5-di-*O*-isopropylidene-α-D-apio-L-furanose, that is, 1,2:3,3[1]-di-*O*-isopropylidene-[3-*C*-(hydroxymethyl)-β-L-threofuranose] (**1**). Graded, acid hydrolysis of **1** gave the 1,2-isopropylidene acetal **6**, which was also synthesized by hydroxylation of 3-deoxy-1,2-*O*-isopropylidene-3-*C*-methylene-D-*glycero*-tetrose (**4**) with osmium tetraoxide. This reaction was highly stereoselective, in that crystalline **6** was formed to the apparent exclusion of its epimer, 1,2-*O*-isopropylidene-α-D-

*For Part I, see ref. 1.

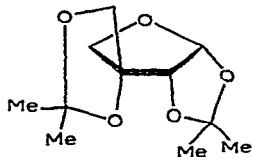
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†Present address: Aldrich Chemical Company, Inc., 2371 North 30th Street, Milwaukee, Wisconsin 53210, U. S. A.

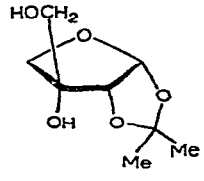
apio-D-furanose (**3**), which would arise by addition of osmium tetraoxide from the more-hindered side of the double bond, namely, *cis* to the isopropylidene ring. Similar steric control was observed in the borohydride reduction of 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**10**), which afforded 1,2-*O*-isopropylidene-D-erythrose exclusively.



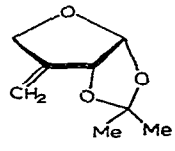
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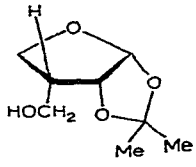
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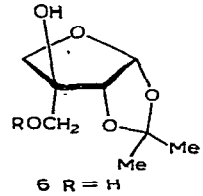
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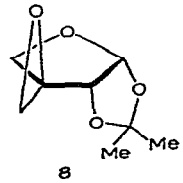
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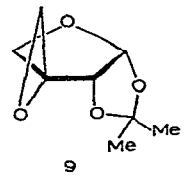
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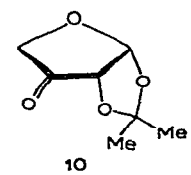
6 R = H
7 R = Ts



8



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This paper describes the preparation of the second isomer of di-*O*-isopropylidene-D-apiose, namely, 1,2:3,5-di-*O*-isopropylidene-α-D-apio-D-furanose, that is, 1,2:3,3[1]-di-*O*-isopropylidene-[3-*C*-(hydroxymethyl)-α-D-erythrofuranose] (**2**), the corresponding 1,2-isopropylidene acetal (**3**), and stereoselective syntheses of 3,5-anhydro- and 3-deoxy-D-apiose derivatives.

DISCUSSION

Pure D-apiose was conveniently prepared from the 1,2-isopropylidene acetal **6** by stirring an aqueous solution, heated to 50°, with washed, cation-exchange resin in the acid form, followed by filtration, and lyophilization of the filtrate. The chromatographically homogeneous product was condensed with acetone containing sulfuric acid, and the course of the reaction was followed by g.l.c. and by t.l.c. In addition to the known diisopropylidene acetal (**1**), a second component of the reaction mixture, having similar chromatographic mobilities, was observed. If the condensation was allowed to reach equilibrium, this component was no longer present, but, by neutralizing the mixture after about 90 min, followed by fractionation

on silica gel, a complete separation of the two major products was achieved. The faster-moving and preponderant component was found to be the isomer **1** previously characterized. The other component, which also crystallized, had physical properties consistent with its formulation as the alternative isomer, 1,2:3,5-di-*O*-isopropylidene-α-D-apio-D-furanose (**2**).

Graded hydrolysis with acid, under the conditions used[1] for the preparation of **6** from **1**, also gave a crystalline 1,2-isopropylidene acetal (**3**), which was different, by p.m.r. spectroscopy and mixture m.p., from **6**.

The antibiotic cordycepin was originally thought to be the adenine nucleoside of 3-deoxy-D-apiose[2,3], but subsequent work has clearly established that its structure is 9-(3-deoxy-β-D-*erythro*-pentofuranosyl)adenine[4]. It would, however, be of interest to devise a simple test to differentiate 3-deoxy-D-apiose and 3-deoxy-D-*erythro*-pentose, in order to avoid possible future confusion. The olefinic sugar **4**, described previously[1], appeared to be a likely intermediate for the preparation of 3-deoxy-D-apiose derivatives*, since anti-Markownikov hydration would generate the desired skeleton. Hydroboration has been successfully applied to carbohydrates for effecting such transformations[5], and it offers the advantage that, where necessary, hindered boranes may be used for increasing the stereoselectivity of the addition.

Treatment of **4** with diborane in tetrahydrofuran, followed by oxidative, alkaline hydrolysis, afforded 3-deoxy-3-*C*-(hydroxymethyl)-1,2-*O*-isopropylidene-α-D-erythrofuranose (3-deoxy-1,2-*O*-isopropylidene-α-D-apio-L-furanose, **5**) as a colorless syrup (in 68% yield), which was characterized as its crystalline *p*-phenylazobenzoate. Analysis (g.l.c.) of the reaction mixture indicated a stereoselectivity of hydroboration (even with this unhindered borane) of at least 95%. The p.m.r. spectrum of **5** clearly showed that, as anticipated, the addition of diborane to the double bond had occurred *trans* to the isopropylidene ring. The H-2 signal appeared as a triplet having a spacing of 4 Hz. For H-2 to be coupled equally with H-1 and H-3, it must be *cis* to both, since the coupling constant between H-2 and H-3 is quite small (<0.5 Hz) when they are *trans* in similar systems[7]. We have found that, for the structurally analogous 1,2-*O*-isopropylidene-D-erythrose, H-2 also gives a triplet ($J_{1,2} \approx J_{2,3} \approx 4$ Hz), whereas, for 1,2-*O*-isopropylidene-D-threose, H-2 gives a doublet ($J_{1,2} \approx 4$ Hz, $J_{2,3} < 0.5$ Hz).

Hydrolysis of **5** with aqueous acetic acid gave 3-deoxy-D-apiose as a pale-yellow syrup which, although not readily separable from 3-deoxy-D-*erythro*-pentose** by paper chromatography, gave a different color-reaction (yellow-brown, compared with dark pink) with the *p*-anisidine hydrochloride spray.

Mono-*p*-toluenesulfonylation of **6** readily gave a high yield of crystalline 1,2-*O*-isopropylidene-5-*O*-*p*-tolylsulfonyl-α-D-apio-L-furanose (**7**), and treatment of **7** with sodium methoxide in methanol at room temperature gave 3,5-anhydro-1,2-*O*-

*Racemic 3-deoxyapiose has been synthesized[6].

**Obtained by hydrolysis of synthetic cordycepin, kindly provided by Drs. E. Walton and F. W. Holly of Merck and Company, Rahway, New Jersey.

isopropylidene-α-D-apio-L-furanose (**8**) as a colorless syrup in 78% yield. The p.m.r. spectrum of **8** was consistent with the *spiro*-epoxide structure and the oxirane-ring protons gave a well-resolved AB system (J = 4.4 Hz) at relatively high field. The same epoxide (**8**) was obtained by treatment of olefin **4** with *m*-chloroperoxybenzoic acid in chloroform. G.l.c. of the reaction mixture indicated that **8** was the major component (96%), and also showed the presence of a minor component (4%). The *spiro*-epoxide **8** was isolated in 52% yield, and was found to be identical with the epoxide prepared from **7**. This result substantiates the previous stereochemical assignments[1], because the sensitivity of peroxy acid oxidations to steric factors is even more well-documented than that of osmium tetraoxide[9].

The minor component from the epoxidation was shown (by g.l.c.) to be 3,5-anhydro-1,2-*O*-isopropylidene-α-D-apio-D-furanose (**9**). The latter was readily synthesized, although in low yield, by transfer of methylene to 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose (**10**). Treatment of **10** with dimethylsulfonium methylide[10] in methyl sulfoxide–tetrahydrofuran gave **9** as a crystalline solid, m.p. 66–67°. The p.m.r. spectrum of **9** was similar to that of the isomeric epoxide **8**, except that the oxirane protons gave rise to a poorly resolved AB system, with little separation of the signals. The mass spectra of the two epoxides were also quite similar; each exhibited virtually no parent ion, and gave intense fragments corresponding to the loss of methyl from the isopropylidene group. This mode of fragmentation is well established for isopropylidene acetals of carbohydrates[13]. The exact, measured mass of the M − 15 peak corresponded closely, in each case, to the value calculated.

It is significant that, whereas dimethylsulfonium methylide reacts with **10** to give **9**, dimethyloxosulfonium methylide produces mainly **8** (86%), together with a minor proportion (14%) of **9**; this result is consistent with those from studies, reported in other contexts[10,11], in which it appears that kinetic control obtains with the sulfonium ylide, and thermodynamic control with the oxosulfonium ylide. In a brief report of the reaction of a glycofuranosulose derivative with the oxosulfonium ylide, no details were given[12].

EXPERIMENTAL*

General. — Solutions were concentrated under diminished pressure. Melting points were determined in glass capillaries with a Thomas–Hoover apparatus, and are corrected. T.l.c. was performed on Kieselgel G, and detection was effected with sulfuric acid. I.r. spectra were recorded with a Perkin–Elmer Model 137 "Infracord" spectrophotometer, and were calibrated against the 1600-cm^{-1} band of polystyrene. Optical rotations were measured with a Bendix–Ericsson ETL–NPL Automatic Polarimeter, and n.m.r. spectra were recorded with a Varian A-60 spectrometer. Peak positions are given in τ units relative to internal tetramethylsilane (τ = 10.00).

*In experimental procedure *A* for the preparation of 1,2-*O*-isopropylidene-α-D-apio-L-furanose 3,5-thionocarbonate, described[1] in Part I, the following should be inserted after the second sentence: "Methyl iodide (0.25 ml, 3.8 mmoles) was added, and the solution was stirred for 30 min at 25° and then boiled for 30 min under reflux."

Gas–liquid chromatographic analyses were performed on a Pye Argon instrument at flow rates of *ca.* 50 ml of argon/min with the following columns: *A*, neopentyl glycol sebacate (10% w/w on 80–100 mesh Chromosorb W, DMCS-AW); *B*, 1:1 mixture of (*a*) Apiezon M and (*b*) diethylene glycol adipate — both 20% w/w on 80–100 mesh Chromosorb W (DMCS-AW).

Preparation of D-*apiose.* — A solution of 1,2-*O*-isopropylidene-α-D-apio-L-furanose[1] (5 g) in water (250 ml) was stirred for 24 h at ~50° with Dowex-50W x8 (H^+) ion-exchange resin (200–400 mesh, 2.5 g) that had been washed with hot methanol and dried. T.l.c. (ethyl acetate) indicated complete hydrolysis. The resin was removed by filtration, and the filtrate was lyophilized to give a clear glass. Paper chromatography (4:1:5 butyl alcohol–acetic acid–water) indicated a homogeneous product.

*Preparation of 1,2:3,5-di-*O*-isopropylidene-α-*D*-apio-*D*-furanose* (**2**). — The product from the hydrolysis of 5 g of mono-*O*-isopropylidene-D-apiose was stirred vigorously with acetone (900 ml), and concentrated sulfuric acid (4.5 ml) was added. After 90 min, anhydrous sodium carbonate (20–30 g) was added, and the suspension was stirred until neutral (~30 min). T.l.c. (ether) indicated two di-*O*-isopropylidene derivatives, together with slower-moving compounds. G.l.c. (column *A* at 160°) indicated that the first two were present in a ratio of *ca.* 4:1, the α-D-apio-L-furanose isomer (**1**) preponderating. The suspension was filtered, the filtrate was concentrated, and the mixture was fractionated by chromatography on a column of Brinkmann silica gel (300 g) with 1:1 ether–hexane as eluant. The two isomers were separated completely. The faster-moving isomer (2.4 g) was identical (by m.p., mixture m.p., and i.r. spectrum) with the previously characterized[1] **1**. The slower-moving isomer (0.50 g), which crystallized from ether–hexane, had m.p. 52.5–54° and $[\alpha]_D^{28}$ +76.5° (*c* 3.0, ethanol); n.m.r. data (chloroform-*d*): τ 4.29 (doublet, $J_{1,2}$ ~3.5 Hz, H-1), 5.69 (doublet, H-2), 5.98 and 6.35 (AB quartet, $J_{4,4'}$ ~8 Hz, H-4,4'); 6.10 (center of a poorly resolved AB quartet, H-5,5'); 8.42, 8.54, 8.61, and 8.65 (3-proton singlets, CMe_2).

Anal. Calc. for $C_{11}H_{18}O_5$: C, 57.38; H, 7.88. Found: C, 57.00; H, 7.98.

Partial hydrolysis of **2**. — A solution of **2** (0.46 g, 2 mmoles) in acetic acid (5 ml) and water (2.5 ml) was kept at room temperature (~28°). The hydrolysis was monitored by t.l.c. (ethyl acetate) and, after 50 h, the major component appeared to be a mono-*O*-isopropylideneapiose (together with **2** and apiose). The solution was concentrated, and the residue was fractionated by chromatography on a column of Brinkmann silica gel (50 g) with ethyl acetate as eluant. Fractions containing the major product were combined, and evaporated to a crystalline residue (0.16 g, 42%) which, after recrystallization from ethyl acetate–hexane, had m.p. 116–118°, depressed to 95–115° by admixture with **6**; $[\alpha]_D^{29}$ +44.0° (*c* 1.0, ethanol); n.m.r. data (pyridine): τ 3.96 (doublet, $J_{1,2}$ ~3.7 Hz, H-1), 5.35 (doublet, H-2), 5.86 and 6.04 (2-proton singlets, C-4 and C-5 protons), 8.44 and 8.64 (3-proton singlets, CMe_2).

Anal. Calc. for $C_8H_{14}O_5$: C, 50.52; H, 7.42. Found: C, 50.50, H, 7.48.

*Hydroboration of 3-deoxy-1,2-*O*-isopropylidene-3-*C*-methylene-*D*-glycero-tetrose*

(4). — A solution of **4** (0.80 g, 5.1 mmoles) in anhydrous tetrahydrofuran (15 ml) was stirred at 25° under an atmosphere of dry nitrogen, and a M solution of diborane in tetrahydrofuran* (6 ml) was added. After 1 h, M sodium hydroxide solution (10 ml) was added, followed by 30% hydrogen peroxide (2 ml). The solution was stirred overnight, tetrahydrofuran was removed by evaporation at 25°, and the residual, aqueous solution was extracted with dichloromethane (3 × 10 ml). The extracts were dried over magnesium sulfate, and evaporation gave 3-deoxy-3-*C*-(hydroxymethyl)-1,2-*O*-isopropylidene-α-D-erythrofuranose (3-deoxy-1,2-*O*-isopropylidene-α-D-apio-L-furanose, **5**) as a clear syrup (0.61 g, 68%). G.l.c. (column *A* at 160°) indicated the product to be at least 95% pure, and, at most, only trace amounts of material isomeric at C-3 could be present. After "Kugelrohr" distillation at 100°/1 torr, the syrup had $[\alpha]_D^{30}$ +38.2° (*c* 2.0, chloroform); n.m.r. data (chloroform-*d*): τ 4.14 (doublet, $J_{1,2}$ ~4 Hz, H-1), 5.29 (triplet, $J_{2,3}$ ~4 Hz, H-2), 5.85–6.40 (poorly resolved multiplet, H-3, 4, and 5), 8.50 and 8.68 (3-proton singlets, CMe_2).

Treatment of **5** (85 mg, 0.5 mmole) in pyridine (1.5 ml) with *p*-phenylazobenzoyl chloride (172 mg, 0.7 mmole) followed by chromatography on Woelm silica gel (30 g) gave crystalline 3-deoxy-1,2-*O*-isopropylidene-5-*O*-*p*-phenylazobenzoyl-α-D-apio-L-furanose (75 mg, 39%). Three recrystallizations from ethanol gave red plates having m.p. 130.5–131.5°, $[\alpha]_D^{30}$ +48.8° (*c* 0.5, chloroform).

Anal. Calc. for $C_{21}H_{22}N_2O_5$: C, 65.96; H, 5.80; N, 7.32. Found: C, 66.18; H, 5.90; N, 7.25.

Hydrolysis of **5**. — A solution of **5** (124 mg, 0.71 mmole) in water (3 ml) and acetic acid (1 ml) was heated for 90 min at 100° and then lyophilized to give 3-deoxy-D-apiose (91 mg) as a light-yellow syrup having $[\alpha]_D^{31}$ +8.2° (*c* 1.6, ethanol). Descending paper-chromatography on Whatman No. 1 filter paper in 3:1:1 butyl alcohol–ethanol–water gave the following *R* values relative to rhamnose: apiose, 0.95; 3-deoxyapiose, 1.2; 3-deoxy-*erythro*-pentose, 1.2. Detection with *p*-anisidine hydrochloride[8] gave yellow-brown spots for apiose and 3-deoxyapiose, whereas 3-deoxy-D-*erythro*-pentose (obtained by hydrolysis of cordycepin) gave a dark-pink color, similar to that given by D-ribose.

Preparation of 1,2-O-isopropylidene-5-O-p-tolylsulfonyl-α-D-apio-L-furanose (**7**). — To a solution of **6** (2.5 g, 13 mmoles) in anhydrous pyridine (25 ml) was added *p*-toluenesulfonyl chloride (3.0 g, 16 mmoles), and the solution was kept overnight at ~25°. Most of the pyridine was removed by evaporation, and the residue was chromatographed on Davison silica gel (grade 950, 60–200 mesh, 150 g) with ethyl acetate as eluant. Fractions containing the product were combined, and evaporated to a crystalline solid. Recrystallization from ether gave the mono-*p*-toluenesulfonate **7** (3.52 g, 78%), having m.p. 137–138°, $[\alpha]_D$ +43.2° (*c* 4.4, chloroform).

Anal. Calc. for $C_{15}H_{20}O_7S$: C, 52.31; H, 5.85; S, 9.31. Found: C, 52.23; H, 5.80; S, 9.33.

*Alfa Inorganics Inc., Beverly, Massachusetts, U. S. A.

Synthesis of 3,5-anhydro-1,2-O-isopropylidene-α-D-apio-L-furanose (**8**). — *A. From* **7**. To a solution of **7** (0.86 g, 2.5 mmoles) in methanol (25 ml) was added sodium methoxide (2.5 mmoles) in methanol (5 ml). After a few h, t.l.c. (ether) indicated complete conversion of the ester into the epoxide. The solution was evaporated, and the residue was extracted with boiling ether. Evaporation of the extracts afforded the epoxide (**8**) as a syrup (0.34 g, 79%). An analytical sample, prepared by "Kugelrohr" distillation at 110°/15 torr, had $[\alpha]_D^{31}$ +67.3° (*c* 1.9, chloroform); n.m.r. data (carbon tetrachloride): τ 4.06 (doublet, $J_{1,2}$ ~4 Hz, H-1), 5.81 (doublet, H-2), 5.70 and 6.44 (AB quartet, $J_{4,4'}$ ~10 Hz, H-4,4'), 6.95 and 7.12 (AB quartet, $J_{5,5'}$ ~4.4 Hz, oxirane-ring protons), 8.53, 8.71 (3-proton singlets, CMe_2).

Anal. Calc. for $C_8H_{12}O_4$: C, 55.81; H, 7.03. Found: C, 55.85; H, 6.87.

The i.r. spectrum was devoid of absorption in the hydroxyl region, and had bands at 925 and 940 cm^{-1} (epoxide). The exact measured mass of the $M-CH_3$ fragment, measured by high-resolution mass spectrometry, was 157.0508. Calc. for $C_7H_9O_4$: 157.0501.

B. By epoxidation of **4**. To a solution of **4** (0.16 g, 1.0 mmole) in chloroform (10 ml) was added *m*-chloroperoxybenzoic acid (0.32 g, 85% pure ≡1.6 mmoles). The solution was kept for 1 week at ~25° and then extracted with saturated sodium hydrogen carbonate solution and dried (magnesium sulfate). Analysis by g.l.c. (column *B* at 175°) indicated that epoxide **8** was the major product (96%), and that a small proportion (4%) of the isomeric 3,5-anhydro-1,2-*O*-isopropylidene-α-D-apio-D-furanose (**9**) was formed (see later). Evaporation of the chloroform solution, followed by distillation of the residue at 110°/15 torr, afforded the epoxide **8**, identical (by g.l.c., t.l.c., and by i.r. and p.m.r. spectroscopy) with that prepared from **7**.

Reaction of 1,2-O-isopropylidene-D-glycero-tetros-3-ulose (**10**) *with dimethylsulfonium methylide.* — Sodium hydride (0.96 g of a 55% dispersion in mineral oil, ≡20 mmoles) was washed with hexane to remove the oil, methyl sulfoxide (7 ml) was added, and the mixture was heated at 60° under nitrogen until evolution of hydrogen ceased. The resulting solution of sodium methylsulfinyl carbanion was diluted with dry tetrahydrofuran (7 ml), and cooled to 0°. A solution of trimethylsulfonium iodide (4.08 g, 20 mmoles) in methyl sulfoxide (14 ml) was added, and the solution was stirred for 1 min. A solution of 1,2-*O*-isopropylidene-D-*glycero*-tetros-3-ulose[1,14] (**10**, 0.63 g, 4 mmoles) in tetrahydrofuran (2 ml) was then added. The bright-red solution that resulted was stirred for 30 min at 0–10° and for 1 h at 25°, and then poured into water (75 ml). The aqueous solution was extracted with ether (5 × 10 ml), and the extracts were combined, washed with water (7 ml), and dried (magnesium sulfate). Evaporation afforded a syrup (0.12 g, 17%) which, after "Kugelrohr" distillation at 140°/15 torr, gave a crystalline product. Recrystallization from hexane gave needles having m.p. 66–67°; n.m.r. data (carbon tetrachloride): τ 4.24 (doublet, $J_{1,2}$ ~4 Hz, H-1), 5.78 (doublet, H-2), 5.92 and 6.44 (AB quartet, $J_{4,4'}$ ~9 Hz, H-4,4'), 7.10 and 7.15 (oxirane ring protons), 8.47 and 8.70 (3-proton singlets, CMe_2).

Anal. Calc. for $C_8H_{12}O_4$: C, 55.81; H, 7.03. Found: C, 56.15; H, 7.12.

The i.r. spectrum of the product was similar to that of **8** and consistent with its formulation as 3,5-anhydro-1,2-*O*-isopropylidene-α-D-apio-D-furanose (**9**). The exact measured mass of the $M \div CH_3$ fragment was 157.0506. Calc. for $C_7H_9O_4$: 157.0501.

Reaction of **10** *with dimethyloxosulfonium methylide.* — To a stirred solution of trimethyloxosulfonium iodide (0.24 g, 1.1 mmoles) in methyl sulfoxide (10 ml) was added sodium hydride (54 mg of a 55% dispersion in mineral oil ≡1.1 mmoles). The solution was stirred under nitrogen for 30 min, a solution of **10** (0.16 g, 1 mmole) in methyl sulfoxide (1 ml) was added, and the solution was stirred for a further 60 min. The solution was then poured into water (50 ml), and the mixture was extracted with ether (3 × 10 ml). The extracts were combined, dried (magnesium sulfate), and evaporated to a syrup. G.l.c. (column *B*) indicated that the epoxide mixture contained 86% of **8** and 14% of **9**.

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OPTICAL ROTATORY DISPERSION OF AMYLOSE FILMS

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ABSTRACT

There is a direct correlation between the structure of amylose in the solid state, as determined by X-ray diffraction, and the conformation of amylose in solution. The specific rotations of amylose films showing a methyl sulfoxide complex (V_{Me_2SO}), amorphous, or alkali–amylose structure are the same as those of amylose in corresponding solutions of methyl sulfoxide, 0.5M potassium chloride, or 0.1–0.5M potassium hydroxide. The rotation of amylose films that give a B-type of X-ray pattern is higher than that of either amylose V or amorphous films; this higher rotation is attributed to the presence of intermolecular hydrogen-bonds between adjacent amylose chains in the B structure. It is also shown that rotatory dispersion data, alone, cannot be used to establish the conformation of amylose in solution, since the parameter λ_c in the Drude equation is insensitive to changes in structure; that is, amylose V_{Me_2SO}, V, and water-soaked B films all have a λ_c of 150 nm. Consideration of X-ray, optical rotation, and published viscosity data leads to the conclusion that there is a continuous transition from a stiff, tightly coiled helix in methyl sulfoxide to a flexible coil in neutral, aqueous solution.

INTRODUCTION

In many cases, optical rotatory dispersion (o.r.d.) can be used to deduce the conformation of a molecule in solution from the parameters of the Drude equation, where $[\alpha][\lambda^2-\lambda_c^2] = K$. For proteins, both parameters, λ_c and specific rotation, have been used to provide some indication of the degree of helical conformation for a given protein molecule in solution[1,2]. To a lesser extent than for proteins, carbohydrate polymers have also been examined by o.r.d. techniques to study molecular conformation in solution, but the results to date have been inconclusive[3–5].

Previous attempts to determine the conformation of amylose in solution have relied on such measurements as viscosity[6–8], light-scattering[6,8], streaming birefringence[9], optical rotation[4], and recently, nuclear magnetic resonance[10] (n.m.r.). The conformation of amylose in the solid state has been established by X-ray diffraction. At least two, basically different conformations exist; these are the helical

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one that gives crystalline V structures, and the extended helix or more linear conformation that crystallizes as A, B, or C structures[11–17].

The conventional manner for making amylose films is to air-dry a water dispersion of amylose, but this procedure gives unsatisfactory films for the work reported here. The films always show a B-type X-ray diffraction pattern, and they are unsuited for optical studies, owing to cloudiness, birefringence, and strains introduced during drying. Work in this Laboratory has resulted in a procedure that uses solutions of amylose in methyl sulfoxide (Me_2SO) for casting films that are optically clear, without form-birefringence or strain. In addition, not only films having the B structure, but also those having V or amorphous structures, have been cast. In V films, the amylose molecule crystallizes as a tight, six-residue helix with diameters of either 13.0 or 13.7 Å and a repeat distance along the molecular axis of 8 Å. If Kreger's B structure[14] is adopted, the six residues are stretched to 21.2 Å, and the molecule may be depicted as a spiral or as an extended helix. Amorphous films are noncrystalline; the chains are randomly arranged, and, as such, are seemingly the best approximation to amylose in solution.

The development of films having satisfactory optical properties meant that, not only could amylose in a known conformation be investigated, but also, that the way was opened for a correlation between the solution and solid-state structures of amylose, as reported herein.

EXPERIMENTAL

Amylose was isolated from corn starch by the butyl alcohol fractionation procedure of Schoch[18], and films were cast from 20% amylose solutions in methyl sulfoxide. Drying of the films in a vacuum oven above 35° gave an amylose–Me_2SO complex structure (V_{Me_2SO})[12] that is closely related to the normal V amylose structures, whereas, below 35°, amorphous films (or, at times, V films) resulted. Generally, V films were made by immersing V_{Me_2SO} or amorphous films in 85–90% ethanol, and heating them for about 0.5 h at a temperature near the boiling point of the solvent. Both the amorphous and the V films were clear, nonbirefringent, and unoriented. Drying of films cast from 20% solutions of amylose in methyl sulfoxide, at humidities greater than about 40%, gave a B structure. Such films, however, were generally cloudy, and it was difficult to prevent a slight degree of orientation from occurring during the drying process. Clear, nonbirefringent, B films showing no orientation were then produced either from amorphous or V films by keeping the films for 4–5 days in a desiccator containing saturated salt solutions that maintained atmospheres of 93–97% relative humidity. More-complete conversion into the B structure was caused by soaking humidified films in water.

O.r.d. spectra of amylose films were recorded at wavelengths from 600 to 190 nm, with a Cary* Model 60 recording spectropolarimeter. Films were placed

*The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

between two quartz plates in a holder that could be rotated from 0 to 360° in a plane perpendicular to the beam of light. For films that showed even the slightest orientation when examined under polarized light, the optical rotation at a given wavelength changed periodically as the films was rotated from 0 to 360°. These films were not studied further, since we were unable to separate the birefringence from the optical rotation. All of the films used were nonoriented, and showed no form-birefringence, either in the plane of the film or in the direction perpendicular to the plane of the film. Films that were cloudy or had surface roughness were immersed in oil before being placed between the quartz plates. Immersion in oil decreased the lower-wavelength limit to approximately 290 nm, owing to absorption of light by the oil. With smooth, clear films, without oil immersion, and at thicknesses of less than 25 μm, it was possible to measure the o.r.d. spectra down to 190 nm.

The carbohydrate density of the films was determined by dissolving 1-cm-square pieces of film in 10 ml of 1.0M potassium hydroxide and then assaying the solution for the concentration of carbohydrate by the anthrone method[19]. Knowledge of the carbohydrate density of a square-centimeter cross-section of film eliminated the necessity for measuring the thickness of the films, since the specific rotation can be defined as being 10 times the observed rotation divided by the weight of carbohydrate per cm^2 of film.

The o.r.d. spectrum of each film was measured at two film-angles, and the sum of the points was fitted to a one-term, Drude equation by the method of least squares.

I.r. spectra of the amylose films were recorded, by the total reflection method, with a Beckman IR-8 spectrophotometer. The films were clamped to both sides of a 2-mm thick, KRS-5 internal-reflection plate made by the Wilks Scientific Corporation.

RESULTS

The o.r.d. spectrum from 600 to 190 nm for amylose films showing the V, B, V_{Me_2SO}, alkali-amylose, or amorphous structure is a simple dispersion curve that fits a one-term, Drude equation; namely, $[\alpha] = K/(\lambda^2-\lambda_c^2)$, where $[\alpha]$ is the specific rotation at a given wavelength (λ), and the constant λ_c is determined from the slope of a plot of $[\alpha]\lambda^2$ against $[\alpha]$. Fig. 1 shows that in no case was an anomalous dispersion curve obtained, although we measured films in which the amylose molecules were helical, according to their X-ray patterns. The X-ray and optical data for the films are shown in Table I, together with rotation data for the corresponding solutions.

No significant difference was observed between the o.r.d. of amylose films having either the V or amorphous structures and that of amylose in aqueous salt (0.5M potassium chloride solution). Upon immersion in water, both the amylose V and the amorphous films expand and change to the B structure.

The specific rotation of amylose B films is higher than that of other amylose films, and it seems to depend upon the method of film preparation (see Table I). Amylose B films that have been changed to B from either the V or amorphous forms, by humidity treatment alone, have an average $[\alpha]_D$ of 346 $\pm$45° and a slightly higher

λ_c of 160 nm. When these films are placed in water, they expand by 10–15% in cross-sectional area, and their specific rotation after drying increases to an average $[\alpha]_D$ of 432 ±45° for water-soaked, amylose B films. Also, the λ_c value for the water-soaked

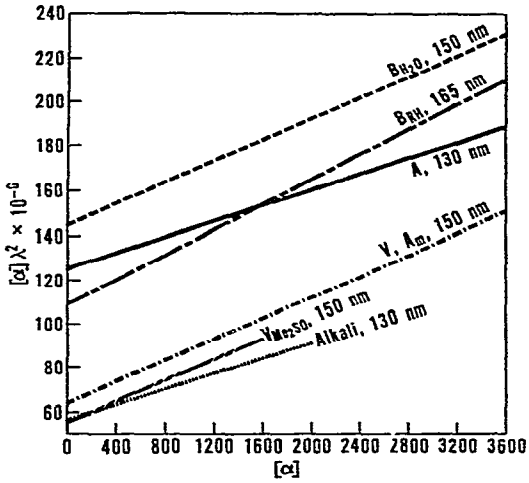


Fig. 1. Modified, Drude plots of the optical rotatory dispersion of amylose B, V, amorphous, V_{Me_2SO} and alkali films, and an amylopectin A film.

TABLE 1

X-RAY PATTERNS AND OPTICAL ROTATIONS OF AMYLOSE AND AMYLOPECTIN FILMS (CORRESPONDING OPTICAL DATA ON SOLUTIONS ARE SHOWN IN THE PARENTHESES)

Sample	*X-ray pattern*[b]	$[\alpha]_D$, *degrees*	λ_c (±3 *nm*)	*Films*[a]
Amylose	amorphous	200 ±10	150	5
(Amylose in 0.5M KCl)		(200)	(148)	
Amylose	V	200 ±10	150	5
Amylose	V_{Me_2SO}	175 ±5	150	2
(Amylose in anhydrous Me_2SO)		(175)	(148)	
Amylose, water-soaked	B	432 ±45	150	10
Amylose, humidity-treated V	B	346 ±45	160	4
Amylose, alkali complex		172 ±10	132	2
(Amylose in 0.5M KOH)		(174)	(130)	
Amylopectin	amorphous	200 ±10	150	2
(Amylopectin in 0.5M KCl)		(200)	(148)	
Amylopectin	A	408 ±63	131	3

[a]Number of films from which average values of $[\alpha]_D$ and λ_c were determined. [b]V, A, and B refer to conventional, X-ray pattern nomenclature for starches. V_{Me_2SO} is an amylose–methyl sulfoxide complex structure, in which the molecules are helical, as in V amyloses.

films apparently decreases to 150 nm. Films that have been made from the B type by soaking in water show no further expansion on reimmersion in water. Expansion

of the films correlates with molecular order within the films, much as swelling measurements correlate with the degree of crosslinking in polymers; in our case, points of association between molecules and the crystallites act as crosslinks. The crystallinity of several films having various proportions of amorphous and B-crystalline structures is shown by the X-ray diffraction patterns in Fig. 2. A greater degree of crystallinity is indicated for the water-soaked B-films than for the humidity-treated films. The caption for Fig. 2 includes rotatory data on the respective films, to show the relationship between $[\alpha]_D$ and film crystallinity.

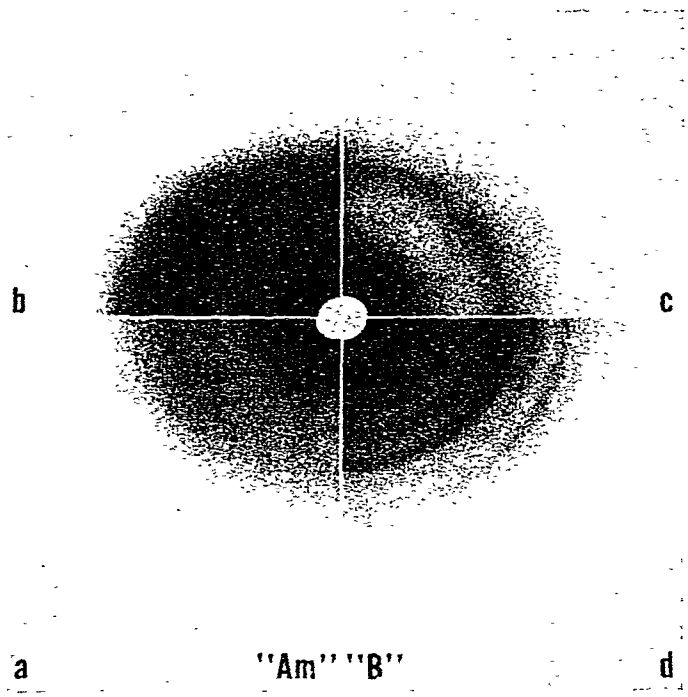


Fig. 2. X-ray diffraction patterns of a series of amylose films having various extents of amorphous and crystalline B structures: (*a*) amorphous film, $[\alpha]_D = 200°$; (*b*) amorphous film plus a trace of B crystalline structure, $[\alpha]_D = 230°$; (*c*) humidity-treated B film, $[\alpha]_D = 376°$; (*d*) water-soaked B film, $[\alpha]_D = 480°$.

The alkali–amylose complex[20] is another amylose structure about which we have, from X-ray studies, information that provides a comparison between the optical rotation of amylose of known structure in the solid state and that of the same sample in solution. Accordingly, amylose V and B films were transformed into the alkali–amylose structure by immersing films in 0.1–0.5M potassium hydroxide in 75% ethanol for 6–9 days. The o.r.d. spectrum of the alkali–amylose films is a simple dispersion, characterized by parameters that are practically identical with the values for the potassium hydroxide solutions.

Films of amylose in the helical conformation, V_{Me_2SO} can be cast directly from

methyl sulfoxide solutions with retention of solvent in the ratio of about 8 methyl sulfoxide molecules per 12 D-glucose residues[12]. The o.r.d. spectrum of the V_{Me_2SO} films (see Table I) is the same as that of amylose in methyl sulfoxide solution. Although these values for amylose in methyl sulfoxide have been reported elsewhere[3], it should be noted that work in this Laboratory, also, has indicated a value of about 190° for the $[\alpha]_D$ of amylose in methyl sulfoxide. The cause of these differences is under investigation. However, as the present study shows, the differences can, in some cases, be ascribed to characterization of an amylose–methyl sulfoxide complex.

Amorphous amylopectin films are characterized by the same o.r.d. curve as that of amylopectin in 0.5M aqueous potassium chloride solution. Amorphous amylopectin films, converted into a crystalline structure by humidifying and water-soaking treatments, show a different λ_c and a much higher $[\alpha]_D$ than before crystallization. We cannot explain the change in λ_c, or the fact that it is significantly lower than other λ_c values for crystalline films. The difference could be due to the A-type crystallinity shown by the amylopectin films, since all of the other water-soaked films have shown the B structure. We were unable to cast a film satisfactory for optical studies from amylopectin having the B structure (in order to compare the two crystal structures from the same material). The experiment did, however, provide another example where crystallization resulted in an increase in specific rotation.

In order to check the o.r.d. of amorphous films further, films made from a β-limit dextrin were used; these have so far shown no evidence of crystallization. The dextrin is the product of the action of *beta*-amylase on amylopectin; presumably, the short side-chains resulting hinder crystallization. Another advantage of this dextrin film was that it could be cast directly from water to give an amorphous form. In so doing, we eliminated the possibility that residual amounts of helical conformations, remaining in amorphous films made from the methyl sulfoxide castings, could account for the measured rotation and λ_c. The β-limit dextrin films, however, gave λ_c and $[\alpha]_D$

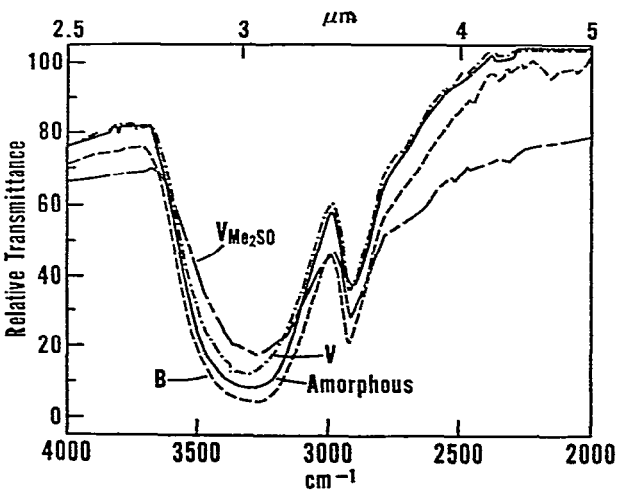


Fig. 3. Infrared spectra of amylose films.

values that were experimentally the same as those observed for the other amorphous samples.

The i.r. spectra of amylose V, V_{Me_2SO}, B, and amorphous films, for the region from 4000 to 2000 cm^{-1}, are given in Fig. 3. It may be seen that, for all of these films, the hydroxyl-stretching frequency is a very broad band centered at approximately 3300 cm^{-1}. An absorption in this region indicates that all of the hydroxyl groups are strongly hydrogen-bonded[21]. There are, however, differences in the width and shape of the band, as is shown in Table II. The width of the band at half-height for the amylose B films is significantly greater than that for the amorphous and V films.

TABLE II

INFRARED SPECTRAL DATA FOR AMYLOSE FILMS

Film of	*X-ray pattern*	*Half-width (in cm^{-1}) of hydroxyl stretching band at 3300 cm^{-1}*	*Slope[a] at short-wavelength side of hydroxyl band*	*Slope at long-wavelength side of hydroxyl band*	*Difference between slopes*
Amylose, water-soaked	B	600	3.95	2.23	1.72
Amylose, humidity-transformed	B	600	4.21	2.27	1.94
Amylose	amorphous	552	3.57	2.48	1.09
Amylose	V	540	3.00	2.53	0.47
Amylose	V_{Me_2SO}	528	1.92	1.50	0.42

[a]Slopes calculated by taking graph units as unity.

Thus, stronger hydrogen-bonding is indicated for the B films[21,22]. In calculating the relative degree of asymmetry of the hydroxyl band, the contribution to the slope at the long-wavelength side of the hydroxyl band, due to a C–H stretching band at 2920 cm^{-1}, was not separated, but was assumed to be the same for all of the films. From the difference in the slopes (see Table II), it may be seen that the band at 3300 cm^{-1} is considerably more asymmetric for the amylose B films as compared with the amylose V and V_{Me_2SO} films. The degree of asymmetry of the hydroxyl band for the amorphous film falls between that for the B and V films.

DISCUSSION

In our work on amylose films, we have shown that it is impossible to establish the conformation of amylose in solution by means of o.r.d. data alone, since the parameter λ_c in the Drude equation is insensitive to structural changes, as evidenced by the fact that V_{Me_2SO}, V, amorphous, and B films all have a λ_c of 150 nm (see Table I). Consequently, the tight helix of the V structure has the same λ_c value as that of the extended form present in the B structure or of the random conformation of

an amorphous film. In examining simpler systems, we, as well as others[3,5], have determined that the cyclic Schardinger dextrins, also, show plain dispersion curves and give λ_c values that are in the range of 148–152, as with the polysaccharides of higher molecular weight. In most cases, the same is true for sugars and simple D-glucosides. Overall, the data on carbohydrates suggest that o.r.d. curves from 600 to 190 nm essentially reflect the dispersion common to the D-glucose residue itself, and that the conformation of the macromolecular chain does not alter the optical dispersion properties. It is not clear why the results for amylose do not parallel observations on proteins or synthetic polypeptides, where anomalous o.r.d. curves are obtained when there is a high degree of helical conformation. Possibly, the behavior is due to the absence from amylose of a strong chromophore, such as is present in the proteins. Beychok and Kabat[5] suggested that a single-term, Drude equation may be expected to fit the o.r.d. data, since the equatorial hydroxyl groups of the D-glucose residue cause weak splitting of relatively weak interactions of hydroxyl dipoles.

Our work has also shown that the o.r.d. of amylose films is generally the same as that of amylose in corresponding solutions. The specific rotation and λ_c of amylose V and amorphous films are the same as those of amylose in aqueous salt (0.5M potassium chloride solution). Similarly, the specific rotations and λ_c values of V_{Me_2SO} and alkali–amylose films are the same as those of amylose in methyl sulfoxide and alkaline solutions, respectively. Further correspondence of specific rotation and λ_c in the solid and the solution states was demonstrated by our obtaining identical measurements on amylose triacetate in chloroform and in film form. These data on solid and solution forms of amylose indicate that, not only polymer–polymer interactions, but also polymer–solvent interactions must be essentially the same. Similar studies on the B structure are somewhat hindered by the fact that amylose cannot be retained in the B form in solution. We observed, however, with the onset of retrogradation of amylose from solution in water, that the optical rotation increased in the same way that it does when films retrograde from the amorphous to the B form. This observation is significant, since it, alone, almost precludes the existence in solution, except for a transient existence, of amylose molecules having the conformation that we know is assumed in the B structure of starch.

For the V_{Me_2SO} and alkali–amylose structures, it has been determined by X-ray analysis that six D-glucose residues repeat in 8 or 23 Å, indicating that the rotation data for a helical and an essentially extended molecular conformation can be compared. The observed low λ_c of 130 and the values for $[\alpha]_D$ (172–175°) are not, however, attributed to conformational changes, but to interactions between the amylose molecule and the alkali or the methyl sulfoxide. This conclusion is supported by the X-ray evidence for complex-formation. Furthermore, the weak, alcohol complexes (V forms) and the amorphous films, from which complexants are absent, show a different but common $[\alpha]_D$ value.

It is clear that the specific rotation is affected by increased molecular association, probably from the effects of increased hydrogen-bonding between amylose chains. This finding is borne out by the corresponding increases in specific rotation and

crystallinity with amylose and amylopectin. The correspondence likewise occurs with the A and B crystalline forms of starch, but not with the helical V structure. This lack of correspondence with V structure argues for intermolecular, not intramolecular, hydrogen-bonding as being partly responsible for the changes in rotation in crystalline films. The reasons for this conclusion are: first, intermolecular association between the more extended molecules in the A and B forms can be expected, and, second, the V form is extensively intramolecularly hydrogen-bonded between the hydroxyl groups on C-2 and C-3′ of adjacent D-glucose residues (according to X-ray diffraction and other studies[13,15,23]). From measurements made on molecular models of amylose in the V form, a distance of 2.8 Å between the C-2 and C-3′ hydroxyl groups is ideally suited for hydrogen bonding of adjacent D-glucose residues on the same chain. On the other hand, assuming Kreger's model for B amylose, the distance between the same hydroxyl groups is 4.2 Å, a value too great for intramolecular hydrogen-bonding, and, hence, there is a great probability for intermolecular bonding.

In summary, the effect of increased molecular association, or of greater crystallinity, is to impart rigidity to the amylose chains, and this results in a higher specific rotation for B- or A-type films. The effect on specific rotation caused by increasing the molecular rigidity is well documented[24].

We conclude, therefore, that complex-formation with the solvent, or the strong interaction between adjacent chains, can perturb electronic transitions and cause changes in λ_c, as well as in [α]. Furthermore, association between chains seems to be more effective in changing the rotation when the molecules are in an extended instead of a helical conformation. Complex-formation results in changes of rotation for either the extended or the helical molecular conformations.

I.r. spectral data for films generally accord with the rotation and other properties determined for the B, V, and amorphous films. For example (see Table II and Fig. 3), the greater width at half-height of the 3300-cm^{-1} absorption band for amylose-B films indicates hydrogen-bonding stronger than for the remaining samples. Likewise, the B-film spectra are skewed slightly toward the lower frequencies that are associated with strong hydrogen-bonds. Correspondingly, the rotations for B films are high, and the films are insoluble in boiling water. The greater asymmetry in the band at 3300 cm^{-1} for the B films over that for the V films suggests that a wider range of hydrogen-bond distances is present in the films. The more symmetrical band in the spectra of V amylose is reasonable on considering that a regularly coiled helix would result in a number of hydrogen bonds between the C-2 and C-3′ hydroxyl groups, having a common distance and, thus, a fairly narrow absorption-band. Taking into account the structure and molecular conformation of the B and V forms, it is possible that an asymmetric band at 3300 cm^{-1} may indicate intermolecular bonding primarily, whereas a symmetrical band would indicate intramolecular hydrogen-bonding. It is of interest, also, that the i.r. data on V amyloses, much like those for other intramolecularly hydrogen-bonded molecules, depart from the linear relationship that has been observed between $\Delta\nu$ cm^{-1} and $\nu_{0.5}$ for intermolecular, hydrogen-bonding systems[22].

Finally, on the basis of our findings and the transformations that take place in amylose films having V, amorphous, or B structures, we consider the question of whether the tight, helical conformation, such as occurs in V amylose, can exist in aqueous solutions. Rao and Foster[4] concluded that, in neutral aqueous solution, amylose exists as a stiff coil having a helical backbone, whereas, at pH 12.0, the helical backbone is partially untwisted because of ionization of hydroxyl groups. Thus, essentially, the amylose molecule undergoes a transition from a stiff coil to a flexible, random coil. Their conclusions[4] were based on the fact that, at pH 12.0, amylose shows a minimum in its intrinsic viscosity, accompanied by a decrease in the optical rotation. On the other hand, monosaccharides show a similar drop in rotation in alkali solutions[25]; and, since the rotatory dispersion of alkali–amylose films is the same as that of amylose in alkali solution, it is apparent that the decrease in optical rotation is characteristic of the alkali complex, and is not necessarily due to increased flexibility of the amylose molecule.

That, in the solid state, amylose has the helical form is clearly established by the X-ray analysis of V_{Me_2SO} or V structures[11,12]. For solutions, the o.r.d. data are practically identical for amylose-V_{Me_2SO} films and for amylose dissolved in methyl sulfoxide. From n.m.r. studies on amylose–methyl sulfoxide solutions, Casu and Reggiani[23] suggested the presence of a hydrogen bond between the hydroxyl groups on C-2 and that on C-3′ of adjacent D-glucose residues in the *Cl*(D) conformation. From examination of scale models of the amylose molecule, it is evident that such a bond imparts a helical twist to the chain. Consequently, both in the solid state and in solution, amylose in methyl sulfoxide probably exists in a relatively stiff, helical conformation.

As shown by Cowie[7], addition of water to amylose–methyl sulfoxide solutions causes a decrease in the intrinsic viscosity, with a minimum at 3:1 water–methyl sulfoxide. The intrinsic viscosity at the minimum is less than that of amylose in 0.5M potassium chloride, whereas the exponent "*a*" in the Staudinger–Kuhn equation is greater. From these results, Cowie concluded that amylose has a more extended form in 0.5M potassium chloride than in methyl sulfoxide. From viscometric and light-scattering data, Banks and Greenwood[8] calculated the length of the Kuhn statistical segment (*L*), which is a measure of the degree of stiffness of a molecule; they showed that *L* for amylose in Me_2SO is 95 Å, as against 21 Å for amylose in neutral, aqueous solution. The lower value of *L* means that the amylose molecule is more flexible in the latter solvent[8,26]. It is evident that amylose in methyl sulfoxide has the properties of a stiff coil, and that this stiffness seems to arise from the presence of helical segments of unknown length. Changes in viscosity resulting from dilution of the methyl sulfoxide solution with water suggest a gradual diminution of the proportion of helical conformation. A corresponding series of transitions is indicated by the X-ray patterns of V films that have adsorbed increasing proportions of water. The transitions can be summarized as follows: $V_{Me_2SO} \rightarrow V \rightarrow$ Amorphous $\rightarrow$ Amorphous + B $\rightarrow$ B. The amorphous intermediate between the helical (V) and the extended form (B) was also reported by Zaslow and Miller[17].

Thus, the instability of the V form towards water, together with the optical rotation and viscosity measurements, leads to the conclusion that the existence of a helical conformation for amylose in neutral, aqueous solutions is highly improbable. The flexible, random-coil form in solution, like the amorphous form in films, is transitory, and readily reverts to either a helical or an extended molecular conformation. The extended form quickly retrogrades and is precipitated from solution, but, as the optical and X-ray results show, these changes occur only from the intermediate, random (amorphous) form, and not directly from the helical conformation. Our overall conclusion is that a flexible, random coil, limited only by restraints imposed by α-D-(1→4)-glucosidic bonds, is the most probable form for amylose in neutral, aqueous solutions.

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SYNTHESES OF 6-DEOXY-2,4- AND 3,4-DI-*O*-METHYL-D-ALLOSE

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ABSTRACT

Two syntheses of 6-deoxy-2,4-di-*O*-methyl-D-allopyranose (**11**) are described. One route utilised the methylation of methyl 6-deoxy-β-D-allopyranoside 3-carbanilate (**4**) which occurred with concomitant migration of the ester group to give a mixture of methyl 6-deoxy-2,4-di-*O*-methyl-β-D-allopyranoside 3-(*N*-methylcarbanilate) (**5**) and methyl 6-deoxy-2,3-di-*O*-methyl-β-D-allopyranoside 4-(*N*-methylcarbanilate) (**6**). The mixture of **5** and **6** was treated with lithium aluminium hydride to give methyl 6-deoxy-2,4- and 2,3-di-*O*-methyl-β-D-allopyranoside (**7** and **8**) which were separated on silica gel and hydrolysed with acid to yield the free sugars **11** and **12**, respectively, which were characterised as the crystalline β-diacetates **13** and **14**, respectively. An unequivocal synthesis of **11** was accomplished by a series of transformations on methyl 3-*O*-benzyl-2-*O*-methyl-α-D-allopyranoside (**15**). Methyl 6-deoxy-3,4-*O*-isopropylidene-β-D-allopyranoside (**21**) was converted into methyl 6-deoxy-3,4-di-*O*-methyl-β-D-allopyranoside (**27**) by sequential benzylation, graded hydrolysis with acid, methylation, and hydrogenolysis. Acid hydrolysis of glycoside **27** liberated 6-deoxy-3,4-di-*O*-methyl-D-allopyranose (**28**) which was characterised as the crystalline β-diacetate (**29**).

The reaction of methyl 6-deoxy-α-L-mannopyranoside with triphenylboroxole yielded a cyclic benzeneboronic ester having the 2,3-structure **31**.

INTRODUCTION

Until comparatively recently, naturally occurring derivatives of D-allose were unknown, and Angyal[1] has commented "It is curious, however, that allose, of only slightly higher free energy (than glucose, mannose, and galactose), seems to be unknown in Nature". Whereas this situation, as far as we are aware, still exists for D-allose, 6-deoxy-D-allose and certain of its methyl ethers have been found in natural products, where they often form a part of molecules of considerable biological interest. Both 6-deoxy-D-allose and its 2-methyl ether (D-javose) occur in the cardiac glycosides present in the seeds of *Antiaris toxicaria* Lesch[2], and 6-deoxy-2,3-di-*O*-methyl-D-allose (D-mycinose) is a component of the antibiotics chalcomycin[3] and tylosin[4]. Syntheses of these deoxy sugars have been reported[5–8]. 6-Deoxy-3-*O*-

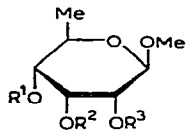
methyl-D-allose has also been synthesised[9,10], and it is now known to occur in a number of plant sources, such as the seeds of *Dregea volubilis*[11] and *D. abyssinica*[12], the roots of *Gongronema taylorii*[13], and the leaves of *Marsdenia erecta*[14]; other plant sources are known[15,16]. 6-Deoxy-4-*O*-methyl-D-allose is known synthetically[8].

The presence of glycosidically bound 6-deoxy-D-allose and D-javose among the cardenolides isolated from the seeds of *Antiaris toxicaria* Lesch[2] is particularly interesting, since exudates of this plant possess marked cardiotonic activity and were used by the Javanese as a poison for blow darts. The rare sugars of the cardiac glycosides[17,18] have no physiological activity of their own but are believed to influence the absorption and distribution of the drugs. There are indications[18] that the carbohydrate component contributes to an adsorptive process that promotes the affinity of the cardiac tissue for the cardenolide. Certain trends that have been noted[18] for structure–activity relationships, in terms of the carbohydrate component, have accrued from studies with a series of cardenolides having minimal structural variations in the glycosidically bound sugar. Glycosides of digitoxigenin and strophanthidin, for example, have been prepared, in which either one or more of the hydroxyl groups was replaced by a deoxy function or the configuration of an asymmetric centre was altered. Cardenolides containing glycosidically bound 6-deoxy-β-D-allopyranose[2,19], 2-deoxy-β-D-*ribo*-hexopyranose[20], and 2,6-dideoxy-D-*ribo*-hexopyranose[21,22] have been shown to possess fairly potent, but varied, cardiotonic activity, and it was felt that an examination of the activity of a series of cardenolides containing methylated derivatives of 6-deoxy-D-allose might provide fresh information on the structure–activity relationship. Apart from certain of the methyl ethers of 6-deoxy-D-allose described above, the dimethyl ethers **11** and **12** were required for this study, and their syntheses are reported below.

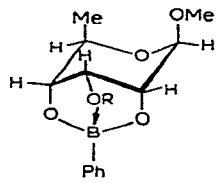
DISCUSSION

The first approach to a synthesis of 6-deoxy-2,4-di-*O*-methyl-D-allose (**11**) was based upon methyl 6-deoxy-β-D-allopyranoside 2,4-benzeneboronate (**2**) which is formed[9] by treatment of alloside **1** with triphenylboroxole in boiling benzene. The structure of the benzeneboronate was inferred[9] from the fact that methyl β-D-ribopyranoside gave a 2,4-boronic ester under analogous conditions[23]. Proof of the structure assigned was furnished by conversion of **2**, by successive reactions with phenyl isocyanate and propane-1,3-diol[23], into a crystalline carbanilate that did not reduce the periodate ion. It is, therefore, methyl 6-deoxy-β-D-allopyranoside 3-carbanilate (**4**), and it must have been derived from the 2,4-boronate **2**. The structure of carbanilate **4** was also indicated by n.m.r. spectroscopy which showed, from the appearance of H-1 as a wide doublet ($J_{1,2}$ *ca.* 8 Hz) at τ 5.56, that the molecule assumed the *C*-1 conformation in solution. It is known[24] that esterification of pyranoside hydroxyl groups invariably produces a downfield shift of the resonance of a proton attached to the same ring-carbon atom, a feature that has provided valuable information on the structures of partially esterified pyranosides. The n.m.r. spectrum of carbanilate **4** showed a narrow triplet (*J ca.* 3 Hz), downfield (τ 4.69)

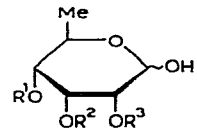
from the H-1 resonance, which could only be assigned to H-3, since this proton is uniquely situated in an equatorial orientation in the *C*-1 conformation. Thus, it is resonable to assume that the C-3 hydroxyl group is esterified.



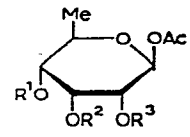
1 $R^1=R^2=R^3=H$
4 $R^1=R^3=H$, $R^2=CONHPh$
5 $R^1=R^3=Me$, $R^2=CON(Me)Ph$
6 $R^1=CON(Me)Ph$, $R^2=R^3=Me$
7 $R^1=R^3=Me$, $R^2=H$
8 $R^1=H$, $R^2=R^3=Me$
9 $R^1=R^3=Me$, $R^2=CONHPh$
10 $R^1=CONHPh$, $R^2=R^3=Me$



2 R = H
3 R = CONHPh



11 $R^1=R^3=Me$, $R^2=H$
12 $R^1=H$, $R^2=R^3=Me$



13 $R^1=R^3=Me$, $R^2=Ac$
14 $R^1=Ac$, $R^2=R^3=Me$

The reaction of **1** with triphenylboroxole parallels that of methyl β-D-ribopyranoside[23] where, although *cis*-vicinal diols are present at the 2,3- and 3,4-positions, the 2,4-positions are preferentially esterified. Although this result accords with observations[25] that six-membered boronate rings are more stable than five-membered boronate rings, Ferrier and Prasad[23] have suggested that, for the riboside 2,4-benzeneboronate, the main stabilising influence results from coordination of the hydroxylic oxygen to the boron atom. Internal coordination of this sort could account for the preferential formation of alloside benzeneboronate **2**, and, incidentally, would lead to a decrease in nucleophilicity of the hydroxyl group at C-3. This might explain our previous failure[9] to methylate this hydroxyl group and its unreactivity towards oxidation by acetic anhydride–methyl sulphoxide[26].

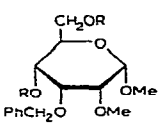
Methylation of carbanilate **4**, by using methyl iodide and silver oxide in *N*,*N*-dimethylformamide, gave a mixture of two products which were shown subsequently to be methyl 6-deoxy-2,4-di-*O*-methyl-β-D-allopyranoside 3-(*N*-methylcarbanilate) (**5**) and methyl 6-deoxy-2,3-di-*O*-methyl-β-D-allopyranoside 4-(*N*-methylcarbanilate) (**6**). It is clear that, concomitant with methylation, migration of the phenylcarbamoyl group to C-4 must have occurred*. Methylation of carbohydrates partially esterified by phenylcarbamoyl groups has been achieved[23,28] under comparable conditions without migration of the ester group, but Bouveng[29] has reported instances of migration of phenylcarbamoyl groups during methylation. Reductive removal (lithium aluminium hydride)[29] of the ester groups from the mixture of **5** and **6** gave the dimethyl allosides **7** and **8**, which were separated by chromatography on silica gel. A crystalline carbanilate **10** was prepared from **8**, whereas the carbanilate **9** derived from **7** was not crystalline. The n.m.r. spectrum of carbanilate **9** did, however, provide the first indication of the structure of the parent compound **7**, since it exhibited a narrow triplet (*J ca.* 3 Hz) at lower field than the H-1 resonance,

*In retrospect, methylation of **4** with diazomethane–boron trifluoride etherate, as described recently[27] for partially acetylated sugars, might take place without migration of the ester group.

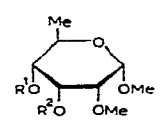
which, for reasons already discussed, suggested that the C-3 hydroxyl group was esterified.

The structure of dimethyl alloside **8** was revealed when, on acidic hydrolysis, it gave a reducing sugar indistinguishable (n.m.r. spectroscopy and t.l.c.) from 6-deoxy-2,3-di-*O*-methyl-D-allopyranose (D-mycinose)[6] (**12**). This identity was conclusively established by acetylation of the free sugar to give the known[30] crystalline *β*-diacetate **14**. Acidic hydrolysis of the isomeric dimethyl alloside **7** afforded syrupy 6-deoxy-2,4-di-*O*-methyl-D-allopyranose (**11**), which was characterised as the crystalline *β*-diacetate **13**. The structures of **7** and **13** were established by their unequivocal synthesis by the route reported below.

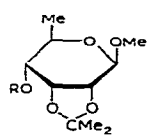
In view of the complication caused by migration of the phenylcarbamoyl group during methylation of carbanilate **4**, an unambiguous synthesis of **11** was devised from methyl 3-*O*-benzyl-2-*O*-methyl-α-D-allopyranoside (**15**). The latter compound was utilised[7] in a synthesis of D-javose, and it is readily prepared by way of the oxidation[31] of methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside. Treatment of **15** with an excess of toluene-*p*-sulphonyl chloride in pyridine yielded disulphonate **16**, which was transformed into methyl 3-*O*-benzyl-6-deoxy-2-*O*-methyl-α-D-allopyranoside (**17**) following treatment with lithium aluminium hydride in benzene–ether. Methylation of **17** and subsequent hydrogenolysis of the benzyl group afforded dimethyl alloside **19**, which gave the free sugar **11** on acid hydrolysis. The free sugar was identical with one of the sugars obtained by the earlier route, and it yielded the same *β*-diacetate (**13**) on treatment with acetic anhydride in pyridine. N.m.r. spectroscopy of an equilibrated solution of the free sugar **11** in deuteriochloroform revealed that the α- and *β*-D-pyranose forms were present in a ratio of 2:1 (see Experimental), although the addition of a few drops of deuterium oxide produced a substantial increase in the proportion of α-D anomer present. It was noticeable, too, that the resonances due to the methoxyl groups exhibited a slightly more-complex pattern than that observed with the glycosides, presumably due to the presence of both anomers in solution. As well as confirming the general



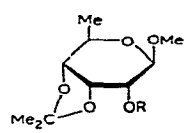
15 R = H
16 R = Ts



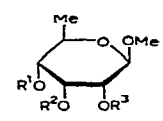
17 R^1=H, R^2=CH_2Ph
18 R^1=Me, R^2=CH_2Ph
19 R^1=Me, R^2=H



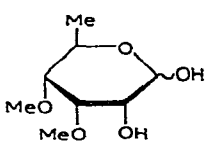
20 R = H
22 R = CONHPh



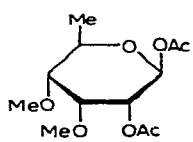
21 R=H
23 R=CONHPh
24 R=CH_2Ph



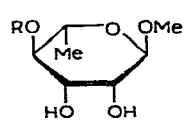
25 R^1=R^2=H, R^3=CH_2Ph
26 R^1=R^2=Me, R^3=CH_2Ph
27 R^1=R^2=Me, R^3=H



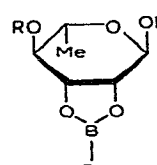
28



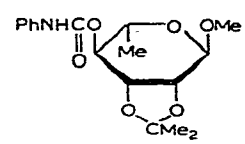
29



30 R = H
33 R = CONHPh



31 R = H
32 R = CONHPh



34

structure of diacetate **13**, n.m.r. spectroscopy clearly indicated the β-D-configuration of the 1-acetoxy group, since the H-1 resonance appeared as a wide doublet ($J_{1,2}$ 8 Hz) at τ 4.20 which was slightly overlapped by a triplet ($J_{2,3} = J_{3,4}$ 3 Hz) at τ 4.14 due to H-3.

6-Deoxy-3,4-di-*O*-methyl-D-allopyranose (**28**) was synthesised by a route involving the acid-catalysed acetonation of methyl 6-deoxy-β-D-allopyranoside (**1**), which is known[8] to yield the 2,3- (**20**) and 3,4-acetals (**21**). It was found that acetals **20** and **21** were readily characterised as the crystalline carbanilates **22** and **23**, respectively. Benzylation of acetal **21** gave methyl 2-*O*-benzyl-6-deoxy-3,4-*O*-isopropylidene-β-D-allopyranoside (**24**), which, on graded, acid hydrolysis and methylation, was converted into methyl 2-*O*-benzyl-6-deoxy-3,4-di-*O*-methyl-β-D-allopyranoside (**26**). Finally, catalytic hydrogenolysis of the benzyl group and acid hydrolysis of the resulting glycoside **27** yielded the syrupy free sugar **28**, which was characterised as the crystalline β-diacetate **29**. N.m.r. spectroscopy showed that an equilibrated solution of **28** in deuteriochloroform contained approximately equal proportions of the α- and β-D-pyranose forms, and this ratio was not altered significantly upon the addition of a few drops of deuterium oxide; the resonances due to the methoxyl and 6-deoxy groups showed as a complex pattern. The β-D-configuration of diacetate **29** was readily seen from its n.m.r. spectrum in which the H-1 resonance appeared as a wide doublet ($J_{1,2}$ 8 Hz) at τ 4.05, well downfield from the other protons.

We also took the opportunity of examining the reaction of methyl 6-deoxy-α-L-mannopyranoside (**30**) with triphenylboroxole in boiling benzene. The condensation yielded a syrupy boronic ester which was converted into a crystalline carbanilate following treatment with phenyl isocyanate. Removal of the benzeneboronate group, with propane-1,3-diol[23], gave a product identified as methyl 6-deoxy-α-L-mannopyranoside 4-carbanilate (**33**), since it was identical with that formed on removal of the acetal group from methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-mannopyranoside 4-carbanilate (**34**). Thus, the original boronic ester is demonstrated to have the 2,3-structure **31**.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed with Kieselgel G (Merck, 7731) with detection by vanillin–sulphuric acid[32]. N.m.r. spectra were measured in deuteriochloroform (tetramethylsilane as internal reference), unless otherwise stated, with either a Perkin–Elmer R-10 or R-14 spectrometer. Infrared spectra were recorded as Nujol mulls (solids) or liquid films on a Perkin–Elmer 'Infracord' spectrometer.

p-Dioxane was dried by distillation from lithium aluminium hydride, *N*,*N*-dimethylformamide by repeated distillation from barium oxide, and benzene and light petroleum fractions were dried over sodium wire.

Methyl 6-deoxy-β-D-allopyranoside 2,4-benzeneboronate 3-carbanilate (**3**). — Benzeneboronate **2** (11 g, m.p. 143–144°, lit.[9], m.p. 145–146°) in dry toluene (400 ml) containing phenyl isocyanate (5 ml) was heated under reflux for 24 h, and the solvents

were removed. The residue was recrystallised from benzene–light petroleum (b.p. 60–80°) to give carbanilate **3** (10.2 g), m.p. 154–155°, $[\alpha]_D$ −74° (*c* 0.7, *p*-dioxane) (Found: C, 62.4; H, 5.8; B, 3.1; N, 4.0. $C_{20}H_{22}BNO_6$ calc.: C, 62.3; H, 5.8; B, 2.8; N, 3.7%).

*Methyl 6-deoxy-β-*D*-allopyranoside 3-carbanilate* (**4**). — To a solution of compound **3** (10 g) in dry acetone (50 ml) was added propane-1,3-diol (5 g), and the solution was set aside for 48 h at room temperature. Removal of the solvents afforded a crystalline residue which was recrystallised several times from ethyl acetate–light petroleum (b.p. 60–80°) to afford alloside carbanilate **4** (4.5 g), m.p. 172–174°, $[\alpha]_D$ −38° (*c* 0.4, *p*-dioxane) (Found: C, 56.3; H, 6.3; N, 4.7. $C_{14}H_{19}NO_6$ calc.: C, 56.6; H, 6.4; N, 4.7%). N.m.r. data (Me_2CO-d_6): τ *ca.* 2.80 (multiplet, 5 aromatic protons), 4.69 (triplet, $J_{2,3} = J_{3,4}$ *ca.* 3 Hz, H-3), 5.56 (doublet, $J_{1,2}$ 8 Hz, H-1), 6.63 (singlet, 3 protons, OMe), and 8.78 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Carbanilate **4** did not reduce sodium metaperiodate in aqueous ethanol under the conditions normally employed[33], and it could be recovered in *ca.* 70% yield after 24 h. T.l.c. also demonstrated that there was no change in the substrate during treatment with periodate ion.

*Methyl 6-deoxy-2,4-di-*O*-methyl-β-*D*-allopyranoside* (**7**) *and methyl 6-deoxy-2,3-di-*O*-methyl-β-*D*-allopyranoside* (**8**). — To a solution of carbanilate **4** (2.5 g) in *N*,*N*-dimethylformamide (40 ml) containing methyl iodide (4 ml) and "Drierite" (5 g) was added freshly prepared silver oxide (10 g) over 6 h, and the suspension was stirred for a further 48 h. Benzene (25 ml) was then added, solids were filtered off, and the solvents were removed to yield a syrup (3 g) which was shown by t.l.c. (acetone–toluene, 1:3) to contain the two components subsequently identified as **5** and **6**. No attempt was made to separate the two components at this stage, but, instead, the mixture (2.88 g) in benzene (20 ml) and ether (240 ml) containing lithium aluminium hydride (2.4 g) was heated under reflux for 3 h to remove the ester group. Ethyl acetate (20 ml) and water (5 ml) were added to decompose the excess of reagent, insoluble material was filtered off and washed thoroughly with ether, and the combined filtrate and washings were concentrated to yield a mobile syrup. Chromatography on silica gel [200 g, elution with ether–light petroleum (b.p. 40–60°), 3:2] gave firstly methyl 6-deoxy-2,3-di-*O*-methyl-β-D-allopyranoside (**8**, methyl β-D-mycinoside) (0.35 g), $[\alpha]_D$ −23 ±2° (*c* 1.1, carbon tetrachloride); attempts to crystallise the glycoside were unsuccessful (lit.[3], m.p. 88–88.5°), but it gave a crystalline 4-carbanilate **10**, m.p. 142–143° [from benzene–light petroleum (b.p. 40–60°)], $[\alpha]_D$ −19 ±1° (*c* 0.5, chloroform), on treatment with phenyl isocyanate as described above (Found: C, 59.1; H, 7.2; N, 4.5. $C_{16}H_{23}NO_6$ calc.: C, 59.1; H, 7.1; N, 4.3%). Continued elution afforded syrupy methyl 6-deoxy-2,4-di-*O*-methyl-β-D-allopyranoside (**7**, 0.51 g), $[\alpha]_D$ −24 ±2° (*c* 2, carbon tetrachloride); n.m.r. data: τ 5.38 (doublet, $J_{1,2}$ 8 Hz, H-1), 5.55 (triplet, $J_{2,3} = J_{3,4}$ *ca.* 3 Hz, H-3), 6.45, 6.56 (singlets, 9 protons, intensity ratio 2:1, 3×OMe), and 8.73 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe). Treatment of **7** with phenyl isocyanate gave a non-crystalline carbanilate **9**, $[\alpha]_D$ −10 ±2° (*c* 0.6, chloroform); n.m.r. data: τ 2.65

(multiplet, 5 aromatic protons), 4.15 (triplet, $J_{2,3} = J_{3,4}$ *ca.* 3 Hz, H-3), 5.43 (doublet, $J_{1,2}$ 8 Hz, H-1), 6.44, 6.51, 6.56 (singlets, 9 protons, 3 × OMe), and 8.69 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

6-Deoxy-2,4-di-O-methyl-D-allopyranose (**11**). — The alloside **7** (0.4 g) in N sulphuric acid (20 ml) was heated on a boiling-water bath for 2 h. The cooled solution was neutralised ($BaCO_3$) and evaporated. A solution of the syrupy residue in ethyl acetate was filtered, and concentrated to give the free sugar **11** as a clear syrup (0.28 g), $[\alpha]_D$ +39° (15 min) → +44° (30 min) → +53° (2 h, equilibrium, *c* 5, chloroform). N.m.r. data ($CDCl_3$): τ 4.82 (broad singlet, H-1, α-D anomer), 5.04 (doublet, $J_{1,2}$ 8 Hz, H-1, β-D anomer), 6.48, 6.52, 6.59 (singlets, 6 protons, 2 methoxyl groups), and 8.73 (doublet, $J_{5,6}$ 6 Hz, CMe); integration gave the ratio of α:β pyranose forms as *ca.* 2:1.

1,3-Di-*O*-acetyl-6-deoxy-2,4-di-*O*-methyl-β-D-allopyranose (**13**), m.p. 142–145° (from ethanol), $[\alpha]_D$ +8 ±1° (*c* 1, chloroform), was prepared by treatment of **11** with acetic anhydride in pyridine. It had m.p. 142–144° on admixture with the diacetate described later (Found: C, 52.5; H, 7.2. $C_{12}H_{20}O_7$ calc.: C, 52.2; H, 7.3%).

Characterisation of 6-deoxy-2,3-di-O-methyl-D-allopyranose (**12**, *D-mycinose*)[3,6]. — Alloside **8** (0.24 g) was hydrolysed, as described above, to give the free sugar **12** (0.13 g) which crystallised slowly but could not be freed entirely from inorganic material. Its n.m.r. spectrum (D_2O) was indistinguishable from that of an equilibrated solution of D-mycinose[3,6]. With acetic anhydride in pyridine, it gave 1,4-di-*O*-acetyl-6-deoxy-2,3-di-*O*-methyl-β-D-allopyranose (**14**), m.p. 101–102° (from hexane–ether), which was shown to be identical (infrared spectroscopy, t.l.c., X-ray powder photograph) with an authentic sample[30]; on admixture, the two samples had m.p. 100–101°.

Methyl 3-O-benzyl-6-deoxy-2-O-methyl-α-D-allopyranoside (**17**). — A solution of methyl 3-*O*-benzyl-2-*O*-methyl-α-D-allopyranoside[7] (**15**, 3 g) in dry pyridine (50 ml) was treated with toluene-*p*-sulphonyl chloride (8 g) in pyridine (50 ml) for 48 h at room temperature. The reaction mixture was then processed in the usual way to give the disulphonate **16** (3.4 g), $[\alpha]_D$ +120 ±2° (*c* 1.7, chloroform), as a clear syrup which also contained a small proportion of a second component presumed to be a monosulphonate. This material was used in subsequent experiments without further purification.

A solution of disulphonate **16** (3.4 g) in benzene (20 ml) and ether (60 ml) containing lithium aluminium hydride (1.5 g) was heated under reflux for 24 h, and the excess of reagent was then decomposed with ethyl acetate (10 ml) and water (2 ml). Solids were filtered off and washed with ether, and the filtrate and washings were concentrated to give a syrup (*ca.* 2 g) containing (t.l.c., acetone–toluene, 1:9) principally one component, together with a small proportion of a second component. Distillation gave essentially pure **17**, b.p. 130–135° (bath)/0.05 mm, $[\alpha]_D$ +110 ±2° (*c* 0.4, chloroform), which was indistinguishable (i.r. and n.m.r. spectroscopy) from a sample prepared by a slightly different route[7].

Methyl 3-O-benzyl-6-deoxy-2,4-di-O-methyl-α-D-allopyranoside (**18**). — Sodium

hydride (1 g) was added to a solution of **17** (1.7 g) in *N,N*-dimethylformamide (60 ml), and the suspension was swirled occasionally for 30 min before methyl iodide (10 ml) was gradually added. After 5 h, the reaction mixture was processed, in the usual manner[34], to yield a syrup which was shown (t.l.c.) to contain a major and a minor component. Chromatography on silica gel (100 g, elution with acetone–toluene, 1:3) separated the major component which was distilled to give dimethyl ether **18** (1.3 g), b.p. 105°/0.05 mm, $[\alpha]_D$ +160° (*c* 1.1, chloroform) (Found: C, 65.5; H, 7.7. $C_{16}H_{24}O_5$ calc.: C, 64.8; H, 8.2%). N.m.r. data: τ 2.70 (multiplet, 5 aromatic protons), 6.60, 6.64, 6.76 (singlets, 9 protons, 3 × OMe), and 8.80 (doublet, 3 protons, $J_{5,6}$ 7 Hz, CMe).

*Methyl 6-deoxy-2,4-di-*O*-methyl-α-*D*-allopyranoside* (**19**). — A solution of **18** (1.1 g) in methanol (60 ml) containing 10% palladised charcoal (0.5 g) was shaken with a slight overpressure of hydrogen for 4 h at room temperature. The catalyst was filtered off, and the solvents were removed to give a residue which afforded D-dimethyl alloside **19** (0.7 g), b.p. 66°/0.05 mm, $[\alpha]_D$ +157° (*c* 0.8, chloroform), on distillation (Found: C, 52.4; H, 8.4. $C_9H_{18}O_5$ calc.: C, 52.4; H, 8.8%). N.m.r. data: τ 5.26 (doublet, $J_{1,2}$ *ca.* 4 Hz, H-1), 6.57, 6.61 (singlets, integrated ratio 1:2, 9 protons, 3 × OMe), 8.74 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Hydrolysis of **19** (0.65 g), as described for the β-D-glycoside **7**, gave 6-deoxy-2,4-di-*O*-methyl-D-allopyranose (**11**, 0.45 g), $[\alpha]_D$ +35.5° (5 min) → +39° (15 min) → +45° (30 min) → +53° (2 h, equilibrium, *c* 1.2, chloroform), which was identical (t.l.c. and n.m.r. spectroscopy) with one of the sugars prepared by methylation of carbanilate **4**.

The derived β-diacetate **13** had m.p. 142–145° (from ethanol), $[\alpha]_D$ +8 ±1° (*c* 1, chloroform), and no depression of melting point was observed on admixture with the diacetate obtained previously. The i.r. spectra and X-ray powder photographs of the two samples were indistinguishable. N.m.r. data: τ 4.14 (triplet, 1 proton, $J_{2,3} = J_{3,4}$ 3 Hz, H-3), 4.20 (doublet, $J_{1,2}$ 8 Hz, H-1, β-D anomer), 6.61, 6.64 (singlets, 6 protons, 2 × OMe), 7.87 (singlets, 6 protons, 2 × OAc), 8.72 (doublet, 3 protons, $J_{5,6}$ 7 Hz, CMe).

*Acetonation of methyl 6-deoxy-β-*D*-allopyranoside* (**1**). — Acetonation of **1** (3 g) was carried out essentially as described by Hoffmann, Weiss, and Reichstein[8]. In our hands, separation of 2,3-acetal **20**, $[\alpha]_D$ −50° (*c* 0.6, chloroform) [lit.[8], −47.4 ±2° (acetone)], and 3,4-acetal **21**, $[\alpha]_D$ −15° (*c* 0.5, chloroform) [lit.[8], −23.4 ±2° (acetone)], on silica gel was best achieved by using butanone–cyclohexane (1:9) as eluant. The n.m.r. spectra of **20** and **21** corresponded to those already reported[8].

Methyl 6-deoxy-2,3-*O*-isopropylidene-β-D-allopyranoside 4-carbanilate (**22**), prepared in the usual way[23], had m.p. 123–124° [from ethyl acetate–light petroleum (b.p. 40–60°)], $[\alpha]_D$ +12 ±1° (*c* 0.9, chloroform) (Found: C, 60.7; H, 6.8; N, 4.3. $C_{17}H_{23}NO_6$ calc.: C, 60.5; H, 6.9; N, 4.15%). The isomeric 2-carbanilate **23** had m.p. 152–153° (from benzene), $[\alpha]_D$ −53° (*c* 0.5, chloroform) (Found: C, 60.3; H, 6.7; N, 3.9%).

*Methyl 2-*O*-benzyl-6-deoxy-3,4-*O*-isopropylidene-β-*D*-allopyranoside* (**24**). —

Sodium hydride (1 g) was added to a stirred solution of acetal **21** (1.2 g) in *N,N*-dimethylformamide (90 ml), and, after 30 min, benzyl bromide (1.4 ml) was gradually added. After 24 h, the excess of reagents was destroyed with methanol (25 ml), and the solution was processed in the usual way[34]. The residue was chromatographed on silica gel (100 g, elution with ethyl acetate–cyclohexane, 1:9) to yield the benzyl ether **24** (1.2 g), $[\alpha]_D$ −16.5 ±1° (*c* 1, chloroform), as a chromatographically homogeneous syrup.

Methyl 2-O-benzyl-6-deoxy-β-D-allopyranoside (**25**). — A solution of acetal **21** (1.2 g) in 50% aqueous ethanol (50 ml) containing glacial acetic acid (12 ml) was heated on a boiling-water bath for 3 h, and t.l.c. (ethyl acetate–cyclohexane, 1:4) then showed that all of the starting material had reacted. Removal of the solvents, with repeated additions of toluene, gave essentially pure **25** (1 g), $[\alpha]_D$ −22 ±2° (*c* 0.9, chloroform), as a clear syrup. N.m.r. data: τ 2.77 (multiplet, 5 aromatic protons), 5.31 (singlet, 2 protons, benzyl methylene protons), 5.36 (doublet, $J_{1,2}$ 8 Hz, H-1), 6.46 (singlet, 3 protons, OMe), and 8.72 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Methyl 2-O-benzyl-6-deoxy-3,4-di-O-methyl-β-D-allopyranoside (**26**). — Methylation of diol **25** (1 g) in *N,N*-dimethylformamide (50 ml) with methyl iodide (10 ml) and sodium hydride (1.5 g) was accomplished as described above. The syrupy residue was freed from unreacted starting material by chromatography on silica gel (100 g, elution with acetone–toluene, 1:19) to give dimethyl ether **26** (1.05 g), $[\alpha]_D$ +11 ±2° (*c* 2, chloroform), as a syrup (Found: OMe, 30.9. $C_{16}H_{24}O_5$ calc.: OMe 31.4%). N.m.r. data: τ 2.72 (multiplet, 5 aromatic protons), 5.26 (AB quartet, *J* 12 Hz, benzyl methylene protons), 5.37 (doublet, $J_{1,2}$ 8 Hz, H-1), 6.41, 6.50, 6.65 (singlets, 9 protons, 3 × OMe), and 8.77 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Methyl 6-deoxy-3,4-di-O-methyl-β-D-allopyranoside (**27**). — A solution of **26** (1 g) in dry methanol (60 ml) containing 10% palladised charcoal (0.5 g) was shaken with a slight overpressure of hydrogen for 4 h at room temperature. Removal of the catalyst and solvents afforded a solid residue which was sublimed at *ca.* 52°/0.05 mm to give the dimethyl alloside **27** (0.7 g), m.p. 50–52°, $[\alpha]_D$ −13° (*c* 1.7, chloroform) (Found: C, 52.3; H, 9.1. $C_9H_{18}O_5$ calc.: C, 52.4; H, 8.8%). N.m.r. data: τ 5.52 (doublet, $J_{1,2}$ 8 Hz, H-1), 6.35, 6.46, 6.53 (singlets, 9 protons, 3 × OMe), and 8.70 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

6-Deoxy-3,4-di-O-methyl-D-allopyranose (**28**). — Glycoside **27** (0.65 g) was hydrolysed in N sulphuric acid (30 ml) as detailed previously. The residue was dissolved in ethyl acetate, solids were filtered off, and the solvents were removed to give the free sugar **28** (0.43 g), $[\alpha]_D$ +48° (5 min) → +50.5° (10 min) → +53° (15 min) → +55° (45 min, equilibrium, *c* 1.5, chloroform), as a chromatographically homogeneous syrup. N.m.r. data ($CDCl_3$ containing 2 drops of D_2O); τ 5.08 (doublet, $J_{1,2}$ 3.5 Hz, H-1 α-D anomer), 5.23 (doublet, $J_{1,2}$ 8 Hz, H-1, β-D anomer), 6.41, 6.43, 6.59, 6.61 (singlets, 6 protons, 2 methoxyl groups), 8.74 and 8.76 (doublets, 3 protons $J_{5,6}$ 6 Hz, C-Me); integration showed an approximately equal ratio of α- and β-D-pyranose forms.

1,2-Di-*O*-acetyl-6-deoxy-3,4-di-*O*-methyl-β-D-allopyranose (**29**), prepared by treatment of **28** with acetic anhydride–pyridine, had m.p. 132–134° (from ethanol), $[\alpha]_D$ *ca.* 0° (*c* 1, chloroform) (Found: C, 52.2; H, 6.9. $C_{12}H_{20}O_7$ calc.: C, 52.2; H, 7.3%). N.m.r. data: τ 4.05 (doublet, $J_{1,2}$ 8 Hz, H-1, β-D anomer), 5.33 (quartet, $J_{1,2}$ 8 Hz, $J_{2,3}$ 3 Hz, H-2), 6.49, 6.61 (singlets, 6 protons, 2 × OMe), 7.94, 7.97 (singlets, 6 protons, 2 × OAc), and 8.75 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Methyl 6-deoxy-α-L-mannopyranoside 2,3-benzeneboronate (**31**). — Methyl 6-deoxy-α-L-mannopyranoside[35] (8 g) in dry benzene containing triphenylboroxole (4.4 g, 0.33 mol.) was heated under reflux for 18 h with collection of water in a Dean and Stark apparatus. Removal of the benzene gave benzeneboronate **31** (11.5 g), $[\alpha]_D$ −34° (*c* 0.9, *p*-dioxane), as a thick syrup. N.m.r. data: τ *ca.* 2.50 (multiplet, 5 aromatic protons), 4.92 (singlet, H-1), 6.50 (singlet, 3 protons, OMe), and 8.65 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Methyl 6-deoxy-α-L-mannopyranoside 2,3-benzeneboronate 4-carbanilate (**32**). — A solution of benzeneboronate **31** (5.9 g) in dry benzene (200 ml) containing phenyl isocyanate (2.5 ml, *ca.* 1 mol.) was heated under reflux for 24 h, and the solvents were then removed. Recrystallisation of the residue from benzene gave carbanilate **32** (1.7 g), m.p. 184–185°, $[\alpha]_D$ +13° (*c* 1, *p*-dioxane) (Found: C, 62.3; H, 6.1; B, 3.0; N, 3.9. $C_{20}H_{22}BNO_6$ calc.: C, 62.3; H, 5.8; B, 2.8; N, 3.7%).

Methyl 6-deoxy-2,3-O-isopropylidene-α-L-mannopyranoside 4-carbanilate (**34**). — Methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-mannopyranoside[36] was treated with phenyl isocyanate, in the usual way, to give an essentially quantitative yield of carbanilate **34**, m.p. 209–210° [from benzene–light petroleum (b.p. 60–80°)], $[\alpha]_D$ −29° (*c* 1, *p*-dioxane) (Found: C, 60.7; H, 6.8; N, 4.5. $C_{17}H_{23}NO_6$ calc.: C, 60.5; H, 6.9; N, 4.15%). N.m.r. data: τ 2.60 (multiplet, 5 aromatic protons), 5.00 (singlet, H-1), 6.53 (singlet, 3 protons, OMe), 8.37, 8.62 (singlets, 6 protons, CMe_2), and 8.74 (doublet, 3 protons, $J_{5,6}$ 6 Hz, CMe).

Methyl 6-deoxy-α-L-mannopyranoside 4-carbanilate (**33**). — (*a*) *From benzeneboronate* **32**. This compound (1 g) in acetone (20 ml) containing propane-1,3-diol[23] (0.25 g, 1 mol.) was kept for 18 h at room temperature, the solvents were removed, and the residue was recrystallised from ethyl acetate–light petroleum (b.p. 60–80°) to give **33** (0.77 g), m.p. 193–194°, $[\alpha]_D$ −86° (*c* 1, *p*-dioxane) (Found: C, 56.6; H, 5.9; N, 4.95. $C_{14}H_{19}NO_6$ calc.: C, 56.6; H, 6.4; N, 4.7%).

(*b*) *From acetal* **34**. This compound (1 g) in glacial acetic acid (7 ml) was heated on a boiling-water bath, and aqueous acetone (3:2, 45 ml) was added in portions over 1 h. Heating was continued for a further 30 min, the solvents were then removed, and the residue was recrystallised (twice) from ethyl acetate–light petroleum (b.p. 60–80°) to give **33** (0.6 g), m.p. 194–195°. It showed no depression of melting point on admixture with the product from (*a*), and the infrared spectra of the two materials were identical.

The products from (*a*) and (*b*) were converted into identical diacetates, which had m.p. 145–146° (from aqueous ethanol), $[\alpha]_D$ −76° (*c* 0.9, *p*-dioxane) (Found: C, 57.0; H, 6.0; N, 3.8. $C_{18}H_{23}NO_8$ calc.: C, 56.7; H, 6.1; N, 3.7%).

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Note

The synthesis of uridine 5′-(3-deoxy-α-D-*ribo*-hexopyranosyl and -4-deoxy-α-D-*xylo*-hexopyranosyl disodium pyrophosphates), and their behaviour in some enzymic reactions

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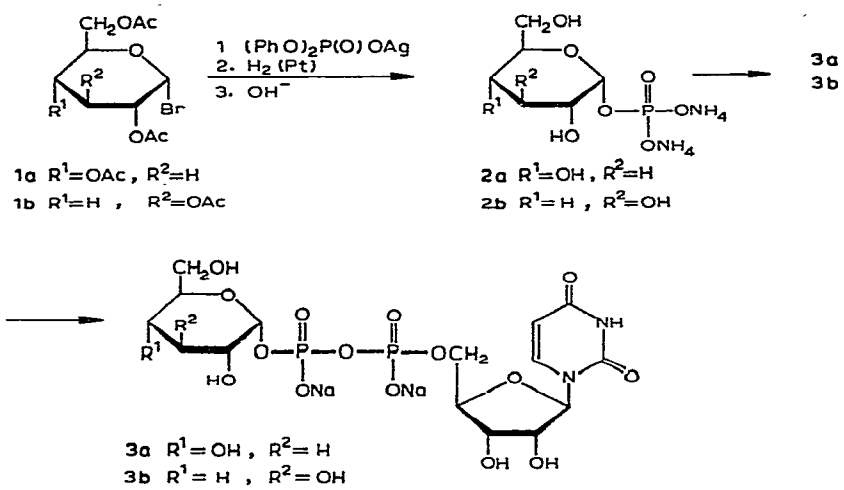
An investigation of the behaviour of nucleoside 5′-(α-D-glycopyranosyl-pyrophosphates) in enzymic reactions is of potential value in attempts to clarify the mechanisms which control the biochemical specificity of these compounds. A study of analogues of uridine 5′-(α-D-glucopyranosyl pyrophosphate) (UDPG) having a modified heterocyclic nucleus[1] has been complemented by the synthesis and investigation of the biochemical properties of UDPG analogues having a modified monosaccharide moiety, *e.g.*, uridine 5′-(α-D-deoxyglycopyranosyl pyrophosphates). We have reported recently the synthesis and some preliminary results of the biochemical investigation of uridine 5′-(6-deoxy-α-D-glucopyranosyl pyrophosphate)[2]. Some analogues of UDPG having modified monosaccharide residues have also been described by Salo *et al.*[3].

We now report the synthesis of two new UDPG analogues modified at C-3 and C-4 of the D-glucose moiety, *viz.*, uridine 5′-(3-deoxy-α-D-*ribo*-hexopyranosyl pyrophosphate) (**3a**) and uridine 5′-(4-deoxy-α-D-*xylo*-hexopyranosyl pyrophosphate) (**3b**). Their behaviour with some of the enzymes involved in UDPG metabolism is also discussed. These include UDPG dehydrogenase (EC 1,1,1,22), catalysing the conversion of UDPG into UDP-glucuronic acid; UDPG 4-epimerase (EC 5,1,3,2), catalysing the reversible transformation of UDPG and UDP-galactose; UDPG: fructose glucosyl transferase (EC 4,1,1,3), catalysing the α-D-glucosylation of fructose; and UDPG:diphenol glucosyl transferase, catalysing the β-D-glucosylation of hydroquinone.

A knowledge of the ability of compounds **3a** and **3b** to participate in UDPG enzymic reactions may be helpful in elucidating the significance of the hydroxyl groups at C-3 and C-4 of the D-glucose moiety in UDPG in relation to biological activity.

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The synthesis of compounds **3a** and **3b** was performed according to the following scheme:



2,4,6-Tri-*O*-acetyl-3-deoxy-α-D-*ribo*-hexopyranosyl bromide[4,5] (**1a**) or 2,3,6-tri-*O*-acetyl-4-deoxy-α-D-*xylo*-hexopyranosyl bromide[4,6] (**1b**) was treated with four equivalents of silver diphenyl phosphate in boiling benzene for 1.5 h. The reaction mixtures were fractionated by using preparative thin-layer chromatography on silica gel (chloroform–acetone, 9:1). The resulting, acetylated glycosyl diphenyl phosphates, without further purification, were hydrogenolysed over platinum, and deacetylated with aqueous ammonia (pH 11.5, 10 h). 3-Deoxy-α-D-*ribo*-hexopyranosyl phosphate (**2a**) and 4-deoxy-α-D-*xylo*-hexopyranosyl phosphate (**2b**) were isolated as ammonium salts after chromatography on a cellulose column (isopropyl alcohol–ammonia–water, 7:1:2).

TABLE I

PAPER-CHROMATOGRAPHIC AND -ELECTROPHORETIC DATA[a]

Substance	*Mobilities* *Paper chromatography*[b]		*Paper electrophoresis*[c]	
	A	*B*	*C*	*D*
α-D-Glucopyranosyl phosphate	—	1.04	1.10	—
2a	—	2.07	1.09	—
2b	—	1.47	1.15	—
UDPG	1.33	—	0.87	1.45
3a	2.07	—	0.89	1.46
3b	1.61	—	0.91	1.47

[a]Mobilities relative to that of uridine 5′-phosphate. [b]Solvent systems: *A*, ethanol–M ammonium acetate pH 7.5 (5:2); *B*, isopropyl alcohol–ammonia–water (7:1:2). [c]Buffers: *C*, 0.05M triethylammonium hydrogen carbonate (pH 7.5); *D*, 0.05M triethylammonium acetate (pH 4.0).

The structures of compounds **2a** and **2b** are supported by the following data. Their electrophoretic mobilities are similar to that of α-D-glucopyranosyl phosphate (see Table I), and the paper-chromatographic mobilities are higher, as may be anticipated. The analytical data correspond to the structure of a sugar monophosphate: the ratio of reducing sugar[7] (after acid hydrolysis) to the acid-labile phosphate[8] was found to be 1.00:1.03 for **2a** and 1.00:0.98 for **2b**. The o.r.d. curves of **2a** and **2b** are similar to that of α-D-glucopyranosyl phosphate and different from that of β-D-glucopyranosyl phosphate (Table II). A similar situation exists with the n.m.r. data (Table II): the position and splitting of the H-1 signals for **2a** and **2b** closely correspond to the H-1 signal for α-D-glucopyranosyl phosphate.

TABLE II

O.R.D. AND N.M.R. DATA

Substance	*Molar rotation*[a] $[\phi] \times 10^{-2}$ (°)				*H-1 signal*[b]			
	500 nm	*400 nm*	*350 nm*	*300 nm*	*Signal form*[c]	*Chemical shift* (p.p.m.)	$J_{1,2}$ (Hz)	$J_{1,P}$ (Hz)
α-D-Glucopyranosyl phosphate	4.15	6.50	9.16	13.4	q	5.53	3.5	7.5
β-D-Glucopyranosyl phosphate	1.21	1.56	2.46	3.55	t	4.94[d]	7.5	7.5
2a	3.65	5.65	7.75	11.4	q	5.48	2.4	7.3
2b	3.95	6.14	8.31	12.2	q	5.45	3.1	7.5

[a]Recorded on a Cary 60 spectropolarimeter; 0.01M solutions in H_2O. [b]Determined at 100 MHz for solutions in D_2O. [c]q, quartet; t, triplet. [d]At 50°.

The triethylammonium salts of **2a** and **2b** were treated with the triethylammonium salt of uridine 5'-phosphomorpholidate[9] (3 equivalents) in anhydrous methyl sulfoxide for 1 h at 60°. Compounds **3a** and **3b** were isolated as the sodium salts by ion-exchange chromatography on Dowex-1 x2 (Cl^-) resin and desalted by gel filtration[10] on Sephadex G-25.

Compounds **3a** and **3b** were homogeneous by paper chromatography and paper electrophoresis, and their mobilities (Table I) were close to those of UDPG. The ratios of reducing sugar[7] (after acid hydrolysis) to acid-labile phosphate[8] and total phosphate[8] were 1.00:1.05:2.03 for **3a** and 1.00:0.98:1.99 for **3b**.

The interaction of compound **3b** with UDPG-dehydrogenase was investigated with an enzyme preparation from calf liver[11]. On incubation of **3b** (0.5 μmole) with NAD (3 μmoles) and the enzyme preparation (1.2 mg of protein) in 0.2M glycine buffer (pH 8.85, 3 ml), practically no change of absorption at 340 nm was observed. This result shows that **3b** was not oxidized, and hence the hydroxyl group at C-4 of the D-glucose moiety is essential for the interaction of UDPG with UDPG-dehydrogenase.

The inability of **3a** to be epimerised with UDPG-4-epimerase isolated from calf liver[12] was shown in the following way. After incubation of **3a** (2.0 μmoles), NAD (0.02 μmole), and the enzyme preparation (200 μg of protein) in 0.2M glycine buffer (pH 8.8, 0.3 ml) for 2 h at 25°, mild, acid hydrolysis of nucleotide sugars was performed, and the resulting hexoses were reduced with sodium borohydride. Analysis of the product by paper chromatography (butyl alcohol–pyridine–water, 6:4:3) showed only 3-deoxy-D-*ribo*-hexitol. No 3-deoxy-D-*xylo*-hexitol, which should be observed if uridine 5′-(3-deoxy-α-D-*xylo*-hexopyranosyl pyrophosphate) was present after the enzymic reaction, was detected. Consequently, **3a** cannot participate in the reaction catalysed by UDPG-4-epimerase.

Compounds **3a** and **3b** were found to be competitive inhibitors of the conversion of UDP-galactose into UDPG, catalysed by UDPG-4-epimerase isolated from calf liver. This reaction was controlled by a method[13] based on the colorimetric determination of D-glucose with glucose oxidase. The inhibition constants were 2.6×10^{-3}M for **3a** and 4.4×10^{-4}M for **3b**.

Compound **3b**, but not **3a**, could be substituted for UDPG in glycosylation catalysed by a UDPG:fructose-glucosyl transferase preparation from pea seedlings[14]. After incubation of **3a** or **3b** (0.8 μmole), fructose (3 μmoles), and the enzyme preparation (400 μg of protein) in 0.5M Tris-HCl buffer (pH 7.3, 0.2 ml), the formation of a fructose-containing disaccharide was demonstrated by a colorimetric determination[15] in the case of **3b**. Free fructose was destroyed by treatment with alkali before application of the colorimetric reaction.

On the other hand, no glycosylation occurs with **3b** in the reaction catalysed by a UDPG:diphenol-glucosyl transferase preparation from wheat germ[16]. No glycoside was detected on the paper chromatogram (butyl alcohol, saturated with water) after incubation of **3b** (0.9 μmole), hydroquinone (2 μmoles), and enzyme preparation (2 mg) in 0.2M Tris–maleate buffer (pH 6.6, 0.2 ml) for 30 min at 37°.

These results show that elimination of the hydroxyl group at C-3 of the D-glucose moiety in UDPG results in the loss of its ability to participate in certain enzyme reactions. After elimination of the hydroxyl group at C-4 of the D-glucose moiety, this ability is retained in only one of the enzymic systems studied.

The first results of biochemical investigation of the analogues prepared demonstrate their potential value for the study of the significance of the structure of the sugar moiety in the biological activity of nucleoside 5′-(α-D-glycopyranosyl pyrophosphates. Further investigations of uridine 5′-(3-deoxy-α-D-*ribo*-hexopyranosyl and 5′-(4-deoxy-α-D-*xylo*-hexopyranosyl pyrophosphates) as substrates or inhibitors of different enzymic reactions of UDPG are in progress in this laboratory.

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Carbohyd. Res., 10 (1969) 152–156

Note

Attempted intramolecular sulphoxide oxidations of alcohols; the diastereoisomeric 1-deoxy-1-ethylsulphinyl-L-galactitols

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Oxidations of alcohols with reagents based on methyl sulphoxide have been used many times with carbohydrate derivatives and are a favoured means for obtaining specific carbonyl compounds[1]. Our interest in stereoselective, enzymic dehydrogenations of alditol derivatives[2], which can be employed in ketose syntheses, led us to investigate the possibility of carrying out comparable dehydrogenations by chemical means with an alcohol–sulphoxide, oxidation–reduction reaction intramolecularly**. Encouragement for this approach came from the known intramolecular participation of sulphoxides in nucleophilic displacements[3], and the consequent knowledge that cyclic sulphoxonium ions of the type required for the success of the proposed reactions can readily be formed.

1-Deoxy-1-ethylsulphinyl-L-galactitol, the enantiomer of which has previously been obtained as a by-product during the ultraviolet irradiation of 1-*S*-ethyl-1-thio-D-galactitol[4], was selected for this study and was prepared by oxidation of 1-*S*-ethyl-1-thio-L-galactitol with hydrogen peroxide in aqueous acetone. Two crystalline, isomeric sulphoxides **1** and **2** were obtained; these differ only in asymmetry at sulphur, since (*i*) on further oxidation, they afforded the same sulphone, and (*ii*) on oxidative cleavage of the diols with periodate, and consequent removal of the carbon asymmetry, residues (presumably ethylsulphinylacetaldehyde) remained which showed enantiomeric o.r.d. curves (Fig. 1).

The absolute configurations at the sulphur atoms have not been determined unambiguously, since the chemical[3b,c,d] and n.m.r.[5] methods which have been employed for characterising sulphoxide stereochemistry are less readily applied in acyclic systems. However, the o.r.d. spectra of the diastereoisomers (Fig. 1) exhibit Cotton effects of opposite sign and with high wavelength extrema near 225 nm. This suggests that isomer **1**, showing the positive effect, has the (*S*) configuration, since (*S*)-1-methylsulphinylbutane also shows a positive Cotton effect in this region[6]. It has to be emphasised, however, that correlation of the alkyl groups attached to the

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***Note added in proof.* Since this paper was submitted it was reported [B. LINDBERG AND H. LUNDSTRÖM, *Acta Chem. Scand.*, 22 (1968) 1861] that the same principle has been applied to methyl-6-deoxy-6-methylsulphinyl-α-D-glucopyranoside. As in the work now described no reaction of the desired type was observed.

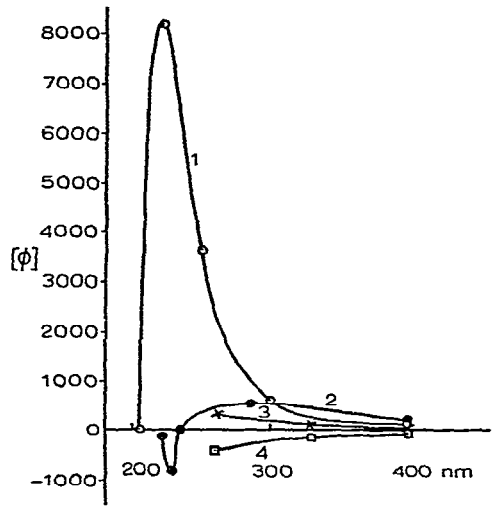
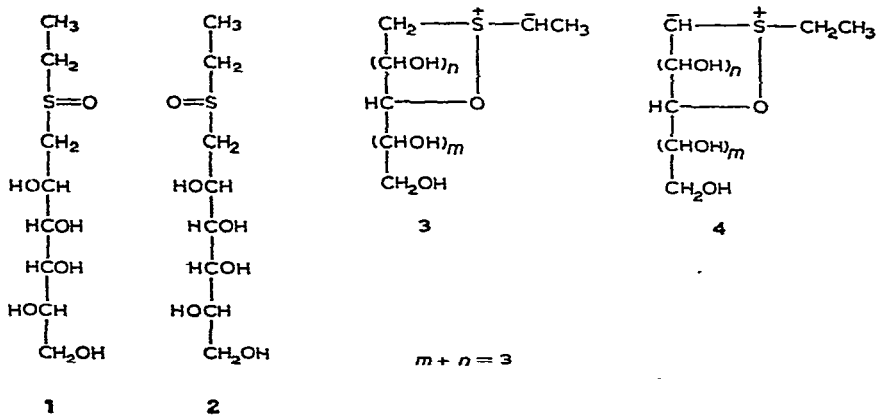


Fig. 1. O.r.d. spectra of 1-deoxy-1-ethylsulphinyl-L-galactitols **1** and **2** (curves 1 and 2, respectively, in ethanol), and their products of periodate oxidation (curves 3 and 4, respectively, in water).

sulphoxide functions on the basis of "size" is hazardous, and that this assignment must be considered to be tentative.

Attempts to carry out intramolecular oxidations of hydroxyl groups with sulphoxide **1**, *N,N'*-dicyclohexylcarbodi-imide, and phosphoric acid [or phosphorus pentoxide or cation-resin (H^+ form)][1] in various solvents and at room or elevated temperatures were uniformly unsuccessful; no indication of the formation of carbonyl products was obtained. Similarly, the use of acetic anhydride and the sulphoxide[1] failed to give carbonyl-containing compounds, but, in some instances, acetylation of hydroxyl groups appeared to occur. It seems probable that the failure of the proposed reaction can be ascribed to the inability on steric grounds of the basic centres in the ylids **3** and **4** to assist in the removal of the carbinol proton as occurs in oxidations with methyl sulphoxide[1]. Whatever the ring sizes of these ylids, neither C-1 of the alditol chain nor C-1 of the ethyl group can readily approach this atom. It is of related interest that the cyclic sulphoxonium ions formed in the sulphoxide-assisted hydrolyses of sulphinylalkyl halides[3a,c] and sulphonates[3b,c,d] cleave hydrolytically and not by loss of the carbinol protons to give ketones[1].



EXPERIMENTAL

1-Deoxy-1-ethylsulphinyl-L-galactitol (**1** *and* **2**). — 1-*S*-Ethyl-1-thio-L-galactitol[7] (10 g) was dissolved in aqueous acetone (150 ml, 70%), and hydrogen peroxide (10 ml, 30%) was added dropwise with shaking. Thin-layer chromatography indicated that the reaction was complete in 3 h, and the solution was then concentrated to *ca.* 15 ml, ethanol (*ca.* 40 ml) was added, and the temperature was decreased to 0°. Two products were then resolved by fractional recrystallisation. Sulphoxide **1** (4.4 g, 41%) was recrystallised from aqueous ethanol and had m.p. 179–180°, $[\alpha]_D$ +85° (*c* 1.2, water), λ_{max}^{KBr} 9.30 μm (sulphoxide), λ_{max}^{EtOH} 213 nm (ε 1080) (shoulder); lit.[4] for the enantiomer, m.p. 178–178.5°, $[\alpha]_D$ −89° (*c* 0.9, water); λ_{max}^{KBr} 9.68 μm, λ_{max}^{EtOH} 226 nm (ε 380). The ultraviolet data recorded here are consistent with those given for methylsulphinylbutane[6] [λ_{max}^{EtOH} 215 nm (ε 1200), shoulder] and for other dialkyl sulphoxides[8] (Found: C, 39.9; H, 7.2; S, 13.7. $C_8H_{18}O_6S$ calc.: C, 39.7; H, 7.5; S, 13.2%). Sulphoxide **2** (1.5 g, 14%) was recrystallised from ethanol–ethyl acetate and had m.p. 126.5–127.5°, $[\alpha]_D$ +16° (*c* 1.0, water), λ_{max}^{KBr} 9.30 μm (sulphoxide), λ_{max}^{EtOH} 213 nm (ε 1070, shoulder) (Found: C, 39.7; H, 7.5; S, 13.2%). The compounds were readily distinguishable by infrared spectroscopy which was used to follow fractional crystallisations; sulphoxide **1** had a sharp absorption at 11.05 μm, whereas this band appeared at 11.30 μm for sulphoxide **2**.

Oxidations of the diastereoisomeric sulphoxides. — (*a*) *With hydrogen peroxide.* The sulphoxides (0.1 g) were separately dissolved in acetic acid (5 ml) containing aqueous hydrogen peroxide (3 ml, 30%) and kept at room temperature for four days, whereupon the solvent was removed to leave residues which crystallised on trituration with ethanol. Recrystallised from ethanol, the sulphone obtained from both sulphoxides had m.p. 160–161°, $[\alpha]_D$ +20° (*c* 1.0, water), λ_{max}^{KBr} 7.75, 9.10 μm (sulphone); the products had identical infrared spectra, and the mixed m.p. was undepressed (Found: C, 37.5; H, 7.1; S, 12.1; $C_8H_{18}O_7S$ calc.: C, 37.2; H, 7.0; S, 12.4%).

(*b*) *With periodate.* The sulphoxides (*ca.* 6 mg) were dissolved in water (0.5 ml), and aqueous sodium periodate (0.5 ml, 0.4M, *ca.* 8 mol) was added; the consumption (determined spectrophotometrically[9]) of oxidant was 4 ±0.2 mol after 5 min, and no further change was detected after 15 min. Excess of periodate was destroyed by the addition of glycerol (100 mg), and the residual, asymmetric compounds formed from sulphoxides **1** and **2** [assumed to be (+)- and (−)-ethylsulphinylacetaldehyde, respectively] were found to have $[\alpha]_D$ values of +48° and −43°, respectively. The o.r.d. curves are shown in the Fig. 1.

Attempted intramolecular alcohol dehydrogenation. — A series of reactions was carried out with sulphoxide **1** (100 mg) and other reagents and solvents (10 ml) as shown, at room temperature and at 60–70° (or reflux temperature for tetrahydrofuran).

Similar experiments were carried out with sulphoxide (1 mol) and phosphorus pentoxide (4.2 mol) in *N*,*N*-dimethylformamide, and with sulphoxide (1 mol) and *N*,*N*′-dicyclohexylcarbodi-imide (5 mol) in *p*-dioxane in the presence of resin [Amberlite IR-120 (H^+)].

Sulphoxide 1 (mol)	1	1	1	1	1	1
N,N'-Dicyclohexyl-carbodi-imide (mol)	2	4	6	5	10	2.5
Phosphoric acid (mol)	0.5	0.5	1	1	1	1.25
Solvent	tetrahydro-furan	$HCONMe_2$	$HCONMe_2$	pyridine	*p*-dioxane[a]	sulpholane–tetrahydro-furan

In none of these experiments were ketonic products detected on chromatograms. Only the initial sulphoxide was detected with a silver nitrate spray; negative tests were given with resorcinol and *p*-anisidine spray reagents. Had the reaction occurred, the sulphur-containing group would have been reduced to an ethylthio group which would have been expected to enhance the chromatographic mobility appreciably.

Treatment of sulphoxide **1** in *p*-dioxane with acetic anhydride caused the formation of components having enhanced chromatographic mobilities, which were believed to be partially acetylated products.

In a control experiment, methyl 4,6-*O*-benzylidene-2-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (kindly supplied by Dr. R. D. King) was successfully oxidised to the appropriate ketone in methyl sulphoxide in the presence of *N,N'*-dicyclohexyl-carbodi-imide and phosphoric acid[10].

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[a] Some sulphoxide remained undissolved in this solvent.

Note

Light-scattering studies of *Acacia* gum exudates

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In a recent article[1], the taxonomic significance of chemical studies of gum exudates from 30 *Acacia* species were assessed. Gum exudates from 10 *Acacia* species in Bentham's[2] Series 4, and from 6 species in his series 5, have been studied to date, and comparisons of the results indicate[1] that these two groups of species are reasonably distinct. Although gum exudates from species in Bentham's Series 1 and 2 have not been studied in any great detail, the indications are that the gums from these species, which are native to Australia, are quite varied. This is not surprising, since many native Australian genera exhibit considerable taxonomic variation and endemism[3]. In these endemic *Acacia* Series, evolution during the long isolation of Australia may have resulted in wider intra-Series divergences than in the pan-tropical groups of Bentham's division of the genus.

Prior to this study, the weight-average molecular weights ($\overline{M}_w$) had been determined (see ref. 1) for only 7 *Acacia* species which are divided between Bentham's Series 1 (*A. cyanophylla*[4]); Series 4 (*A. arabica*[5,6], *A. drepanolobium*[7], *A. nubica*[6], and *A. seyal*[8]); and Series 5 (*A. campylacantha*[9] and *A. senegal*[10,11]). The present study was carried out so that a more comprehensive picture of the *Acacia* genus, with respect to molecular size, could be obtained.

The weight-average molecular weights, $\overline{M}_w$, and the values of *dn/dc* for the *Acacia* gum exudates examined in this study, together with the values which had been reported earlier (*cf.*, ref. 1) are shown in Table I, in which the *Acacia* species are divided according to Bentham's classification[2]. The species studied comprise 4 species in Bentham's Series 1 (PHYLLODINEAE), 4 species in Series 2 (BOTRYOCEPHALAE), 11 species in Series 4 (GUMMIFERAE), and 5 species in Series 5 (VULGARES). Gums from species in Series 3 and 6 have not been studied.

Before this study, the $\overline{M}_w$ of the gums from four species in Series 4 (*A. arabica*[5,6], *A. drepanolobium*[7], *A. nubica*[6], and *A. seyal*[8]) were known to have much higher molecular weights than the two species from Series 5 (*A. senegal*[10,11] and *A. campylacantha*[9]). The new data reported in this Paper agree with these earlier findings. The upper limit for $\overline{M}_w$ of a Series 5 gum is 725,000; only two Series 4 gums have a lower $\overline{M}_w$ than this (*i.e.*, 580,000 for *A. tortilis*, and 625,000 for *A. multijuga*). The average $\overline{M}_w$ for the 11 gums of Series 4 which have now been studied is 1,460,000, and the average $\overline{M}_w$ for the five gums of Series 5 is 590,000. This pronounced difference in $\overline{M}_w$ supports other analytical and structural evidence which indicates that the groups of species in Series 4 and 5 are reasonably distinct.

TABLE I

VALUE OF $\overline{M}_w$ AND *dn/dc* FOR SOME *Acacia* GUMS

		$\overline{M}_w$	dn/dc
Series 1. PHYLLODINEAE	*A. cyanophylla* (S. A.)	470,000	0.146
	A. cyanophylla (W. Aus.)	610,000	0.147
	A. cyanophylla	183,000 (ref. 4)	—
	A. podalyriifolia	74,000	0.146
	A. pycnantha	60,000	0.149
	A. rubida	430,000	0.145
Series 2. BOTRYOCEPHALAE	*A. dealbata*	1,200,000	0.145
	A. elata	70,000	0.145
	A. mearnsii	460,000	0.148
	A. parramattensis	1,220,000	0.146
Series 3. PULCHELLAE	—	—	—
Series 4. GUMMIFERAE	*A. adansonii*	2,360,000	0.146
	A. arabica	2,300,000 (ref. 5)	0.146
	A. arabica	1,870,000 (ref. 6)	0.146
	A. drepanolobium	950,000 (ref. 7)	0.144
	A. fistula	740,000	0.145
	A. giraffae	1,780,000	0.146
	A. karroo	1,460,000	0.145
	A. multijuga	625,000	0.145
	A. nilotica	2,270,000	0.146
	A. nubica	870,000 (ref. 6)	0.147
	A. seyal	850,000 (ref. 8)	0.149
	A. tortilis	580,000	0.147
Series 5. VULGARES	*A. campylacantha*	300,000 (ref. 9)	0.147 (ref. 9)
	A. laeta var. *hashab*	725,000	0.144
	A. laeta var. *mellifera*	670,000	0.145
	A. mellifera	640,000	0.146
	A. senegal	580,000 (ref. 11)	0.146
	A. senegal	600,000 (ref. 10)	0.146
Series 6. FILICINAE	—	—	—

Ten of the Series 4 gum exudates for which $\overline{M}_w$ is now known are from species in Bentham's Sub-series 2 (Medibracteatae); *A. giraffae* is placed in Sub-series 1 (Summibracteatae). More gums from species in Series 4, Sub-series 1 and 3, should be examined to ascertain whether all species in Series 4 exude gum of high $\overline{M}_w$. All five of the Series 5 gums for which $\overline{M}_w$ is now known are from species in Sub-series 1 (Gerontogeae Spiciflorae). Gums from species in Series 5, Sub-series 2, 3, and 4, must be examined before it can be concluded that, on the average, Series 4 gums have higher $\overline{M}_w$ than the Series 5 gums.

In this study, the $\overline{M}_w$ of gum exudates from 4 species in Bentham's Series 1, Sub-series 6 (Uninerves) are reported. The gums from *A. pycnantha* and *A. podalyriifolia* have $\overline{M}_w$ = 60,000 and 74,000, respectively; these are by far the lowest values reported to date for *Acacia* gums, although the surprisingly low $\overline{M}_w$ of *A. pycnantha* gum was predicted from molecular-sieve chromatographic studies[1,12]. Two samples of *A. cyanophylla* gum were examined; a sample from Western Australia had $\overline{M}_w$ higher than of a South African specimen (610,000 compared with 470,000). Both

these values are, however, higher than that obtained by Joubert[4] for a South African sample ($\overline{M}_w$ = 183,000). If Joubert's value is correct, this infers that different gum samples from an *Acacia* species could have $\overline{M}_w$ varying by a factor of three. From a viscosity study, it appeared that the $\overline{M}_w$ of different samples of *A. senegal* gum could vary by a factor of two[13]. It is, however, possible that Joubert's $\overline{M}_w$ for *A. cyanophylla* gum is an underestimation, since his method of clarification (ultracentrifugation) may have removed the larger gum molecules[4].

The wide spread of $\overline{M}_w$ for the Series 1 gums is exceeded by the Series 2 gums. The gum exudates from *A. elata*, *A. dealbata*, *A. mearnsii*, and *A. parramattensis* have $\overline{M}_w$ = 70,000, 1,200,000, 460,000, and 1,220,000, respectively. These results for the gum exudates of Series 1 and 2 agree with the analytical and structural evidence, which indicates that the gums from Series 1 and 2 are quite varied[1].

Although *Acacia* species of Series 1 and 2 are native to Australia, many are now also cultivated in South Africa. Thus, the specimens of the gums from *A. cyanophylla*[14,15], *A. dealbata*[16-18], *A. elata*[14], *A. mearnsii*[14,16,18,19], and *A. podalyriifolia*[14] studied structurally were obtained from African trees. It is well known that the chemical composition of plants is affected by their environment; chemotaxonomic differences could well occur in a species transported from its native habitat to a new environment. The determination in this study of $\overline{M}_w$ for two specimens of *A. cyanophylla* gum from widely different environments is therefore of taxonomic interest; it is perhaps surprising that similar molecular weights were obtained, despite the many uncontrollable seasonal and other differences (such as the age of the trees) that were involved. However, comparative structural studies of these, or of similar specimens from any other *Acacia* species, would give useful results.

Although the number of *Acacia* gums for which $\overline{M}_w$ is known has been greatly increased by this study, only a very small fraction of the gums from the known *Acacia* species have been studied. Many more *Acacia* gums must be studied by the light-scattering technique before the trends suggested by this study can be confirmed.

EXPERIMENTAL

Origins of specimens. — The origins of the following specimens have been given elsewhere: *A. adansonii* Guill. et Perr.[16]; *A. dealbata* Link.[17]; *A. fistula* Schweinf.[17]; *A. giraffae* Willd.[16]; *A. karroo* Hayne[16]; *A. laeta* var. *hashab*[20]; *A. mearnsii* De Wild.[16]; *A. mellifera* (Vahl.) Benth.[16]; *A. nilotica* (L.) Willd. ex Del.[17]; *A. pycnantha* Benth.[16]; and *A. tortilis* Hayne[16]. The Australian sample of *A. cyanophylla* Lindl. was collected by the Curator of the Western Australian Herbarium in April 1968. The South African sample of *A. cyanophylla* Lindl. was collected by Mr. M. J. Wells at Grahamstown, Cape Province, on 22nd February 1968 (Botanical Research Institute of South Africa Voucher Number 3894). *A. podalyriifolia* A. Cunn. ex G. Don and *A. elata* A. Cunn. ex Benth. were collected by Officers of the Botanical Research Institute, Pretoria, in March 1967. The sample of *A. laeta* var. *mellifera* was collected at El Ain Forest Reserve by the Sudanese Gum Research Officer in

December 1960. The specimen of gum from *A. rubida* A. Cunn. was collected by Mr. A. Rodd at Burbong, New South Wales, on 25th April 1968 (Voucher N.S.W. 99697). The specimen from *A. parramattensis* Tindale was collected by Mr. P. Hind at Marayong, Sydney, on 28th July 1968 (Voucher N.S.W. 100274).

Light-scattering measurements were carried out with a SOFICA Photo Gonio Diffusometer, using the methods which have been described previously[6,11]. Determinations were carried out for each sample at three different concentrations (c, $c/2$, $c/4$). The values quoted for $\overline{M}_w$ are the average of three such determinations.

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Note

The preparation of *N*-*p*-nitrophenylglycosylamines of aldodisaccharides

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In the course of preparing some ketodisaccharides from aldodisaccharides by the Lobry de Bruyn–Alberda van Ekenstein transformation, new *N*-*p*-nitrophenylglycosylamines of cellobiose, lactose, and maltose were readily prepared in high yields by treatment in a 50–80% ethanol solution. The preparation of these compounds and their acetates is described in this paper.

The *N*-*p*-nitrophenylglycosylamines of aldoses have generally been prepared by treatment with *p*-nitroaniline in boiling methanol or ethanol, that contained up to 17% of water, in the presence of acetic acid or hydrochloric acid[1–4]. The preparation of crystalline *N*-*p*-nitrophenylgentiobiosylamine required a prolonged heating in absolute methanol, and resulted in some unreacted starting material[2].

During the study of the preparation of *N*-*p*-nitrophenyllactosylamine, we found that the maximum yield was obtained with a 62% aqueous ethanol solution. The yield rose gradually with the increase of the ethanol concentration, but at a concentration of 65% of ethanol, unreacted lactose was found in the precipitate. Obviously, the crystallization of the lactosylamine during the heating and before the complete dissolution of lactose limits the concentration of products that can be used practically. Under the specified condition, lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) did not react with *p*-nitroaniline to form a *N*-*p*-nitrophenylglycosylamine. *N*-*p*-Nitrophenylmaltosylamine was first prepared, probably in amorphous form, in 65% yield by Guthrie and Honeyman[4], but they did not record the physical constants of their specimen. In contrast to previous reports on other substituted maltosylamines[5], *N*-*p*-nitrophenylmaltosylamine dihydrate was found to be an easily recrystallizable product having a sharp melting point. Storage of the glycosylamine derivatives of these disaccharides for a long period without decomposition suggests that these products are as resistant to an Amadori rearrangement as the monosaccharide glycosylamines[6,7].

The disaccharide glycosylamines have been characterized as crystalline heptaacetates having a sharp melting point and an optical rotation devoid of mutarotation.

EXPERIMENTAL

Melting points were determined with a Yanagimoto micro melting-point

apparatus and are uncorrected. Elementary analyses were performed by Misses A. Sato and N. Nanjoh, Pharmaceutical Institute, Tohoku University School of Medicine.

N-p-*Nitrophenyllactosylamine.* — A suspension of α-lactose monohydrate (9 g, 0.025 mole), *p*-nitroaniline (4.5 g, 0.03 mole) in 62% ethanol (20 ml), and glacial acetic acid (0.5 ml) was boiled at reflux for 1 h. The lactose was dissolved within 30 min. After 40 min, crystallization of the product occurred, and ethanol (25 ml) was added to the hot mixture. After being kept overnight at 0°, the crystals were filtered off and washed with a small amount of ethanol and ether. The dried crude product (10.6 g, 88.5% based on lactose) was recrystallized twice from 50% ethanol to give pale-yellow crystals, m.p. 237–238° (decomp.); $[\alpha]_D^{20}$ −66.0° (*c* 1.0, 50% aqueous pyridine). The m.p. was raised to 240–241° with rapid heating. No change of the optical rotation was observed within 24 h.

Anal. Calc. for $C_{18}H_{26}N_2O_{12}$: C, 46.72; H, 5.69; N, 6.06. Found: C, 46.88; H, 5.81; N, 6.05.

The compound kept in a desiccator in the presence of sulfuric acid showed no discoloration during 14 months at room temperature. When the condensation was performed in 50 and 60% of ethanol, the yields were 75 and 83% of the theoretical one, respectively. In 65 and 70% of ethanol, the final product was contaminated with unreacted lactose.

N-p-*Nitrophenyllactosylamine heptaacetate.* — A suspension of *N-p*-nitrophenyllactosylamine (4 g) in pyridine–acetic anhydride (40 ml, 1:1) was shaken for 6 h at room temperature. After 2 days at room temperature, the solution was poured into ice-water. The precipitate was washed thoroughly with cold water and dried. The crude product (6.0 g, 92%) was recrystallized 4 times from ethanol to give colorless crystals, m.p. 232°; $[\alpha]_D^{20}$ −70.8° (*c* 1.5, chloroform).

Anal. Calc. for $C_{32}H_{40}N_2O_{19}$: C, 50.77; H, 5.33; N, 3.70. Found: C, 50.49; H, 5.38; N, 3.78.

N-p-*Nitrophenylcellobiosylamine.* — A suspension of cellobiose (2.0 g, 0.006 mole), *p*-nitroaniline (1.0 g, 0.007 mole) in 50% ethanol (10 ml) and glacial acetic acid (0.2 ml) was heated for 40 min at reflux over a boiling water bath. The solution was then evaporated to a syrup under diminished pressure and kept for a day at room temperature. After being filtered off and washed, the product (3.4 g, 75%) was recrystallized twice from 50% ethanol to give pale-yellow crystals, m.p. 222–223° (decomp.); $[\alpha]_D^{20}$ −164° (*c* 0.6, 50% pyridine).

Anal. Calc. for $C_{18}H_{26}N_2O_{12}\cdot C_2H_5OH\cdot H_2O$: C, 45.60; H, 6.50; N, 5.31. Found: C, 45.50; H, 6.09; N, 5.34.

Under the condition described earlier, no discoloration of this compound was observed.

N-p-*Nitrophenylcellobiosylamine heptaacetate.* — The method of preparation was essentially that used for the lactosylamine acetate. *N-p*-Nitrophenylcellobiosylamine (2.0 g) was treated with pyridine–acetic anhydride (60 ml, 1:1) for 3 days at room temperature. The crude product (3.0 g, 92%) was recrystallized twice from

ethanol–chloroform (7:3) to give colorless needles, m.p. 270–271°, $[\alpha]_D^{20}$ −44.0° (*c* 1.5, chloroform).

Anal. Calc. for $C_{32}H_{40}N_2O_{19}$: C, 50.77; H, 5.33; N, 3.70. Found: C, 49.69; H, 5.25; N, 3.78.

N-p-*Nitrophenylmaltosylamine.* — A suspension of maltose monohydrate (4.5 g, 0.013 mole), *p*-nitroaniline (2.2 g, 0.016 mole) in 80% ethanol (20 ml), and glacial acetic acid (0.3 ml) was heated at reflux for 50 min. The solution was evaporated to a syrup under diminished pressure and kept for several days at room temperature. Generally, the crystallization occurred within 10 days. After being washed, the crude product (5.3 g, 87%) was recrystallized thrice from absolute ethanol to give a yellow crystalline dihydrate, m.p. 152–153°, $[\alpha]_D^{20}$ −44.1° (*c* 1.0, pyridine).

Anal. Calc. for $C_{18}H_{26}N_2O_{12}\cdot 2H_2O$: C, 43.75; H, 6.08; N, 5.62. Found: C, 42.81; H, 6.35; N, 5.33.

No discoloration of this compound was observed under the conditions described earlier.

N-p-*Nitrophenylmaltosylamine heptaacetate.* — A solution of *N-p*-nitrophenylmaltosylamine (3.0 g) in pyridine–acetic anhydride (30 ml, 1:1) was kept for 1 day at room temperature. After being poured into ice-water, it gave a pale yellow powder (3.2 g, 65%) which was recrystallized twice from ethanol–chloroform (15:1) to give colorless crystals, m.p. 220–221°; $[\alpha]_D^{20}$ +12.0° (*c* 1.5, chloroform); lit.[4], m.p. 226°, $[\alpha]_D^{20}$ +14.5° (*c* 0.79, chloroform).

Anal. Calc. for $C_{32}H_{40}N_2O_{19}$: C, 50.77; H, 5.33; N, 3.70. Found: C, 51.08; H, 5.47; N, 3.81.

Treatment of lactulose with p-*nitroaniline.* — A suspension of crystalline lactulose (4.0 g, 0.012 mole), *p*-nitroaniline (2.0 g, 0.014 mole) in 60% ethanol (20 ml), and glacial acetic acid (0.4 ml) was treated under the same conditions as those used for the preparation of *N-p*-nitrophenylmaltosylamine. Unchanged lactulose (3.8 g, 95%) was obtained by adding ether to the reaction product.

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Note

3,5-Dideoxy-3,5-difluoro-D-xylose[1]

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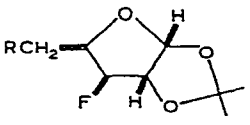
The availability of 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[2,3] and its ready conversion[3] into 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-α-D-glucofuranose and 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-α-D-xylofuranose opens a synthetic route to di- and poly-fluorinated sugar derivatives. We now report the synthesis and characterisation of 3,5-dideoxy-3,5-difluoro-D-xylofuranose, the first example of a difluoro sugar; Kent and co-workers[4] have described two geminal, difluorinated derivatives of alditols, *viz.*, 2,5-anhydro-1-deoxy-1,1-difluoro-D-mannitol and the D-ribitol analogue.

Brief treatment of 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-xylofuranose[3] (**1**) with anhydrous potassium fluoride in boiling ethane-1,2-diol gave four products, of which 3,5-dideoxy-3,5-difluoro-1,2-*O*-isopropylidene-α-D-xylofuranose (**2**), isolated as a syrup by column chromatography, preponderated. The ^{19}F-n.m.r. spectrum (Varian HA-100, 94.01 MHz, CCl_4) of the difluoride **2** showed 8 signals (quartet of doublets) for F-3 (centre of gravity, ∅ +209.8 p.p.m. with respect to CCl_3F) having coupling constants $J_{H-3,F}$ 49.5, $J_{H-4,F}$ 29.9, and $J_{H-2,F}$ 11.0 Hz (*cf.* the values[3] for 3-deoxy-3-fluoro-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose), and 6 signals (triplet of doublets) for F-5 (∅ +231.5 p.p.m.) having $J_{H-5,F}$ 46.0 and $J_{H-4,F}$ 11.9 Hz; there was also a small doublet splitting (~1.4 Hz) and a small triplet splitting (~1.2 Hz) in the signals for F-5 and F-3, respectively, presumably due to long-range coupling[5]. The ^{1}H-spectrum (Perkin–Elmer R10, 60 MHz, CCl_4, internal tetramethylsilane) showed, *inter alia*, a doublet at τ 4.1 ($J_{1,2}$ 4 Hz) for the anomeric proton, and singlets at τ 8.55 and 8.70 for the Me protons of the isopropylidene group. The signal for H-3 was essentially a doublet of doublets with the low-field portion at τ 4.70.

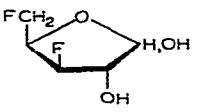
Mild, acidic hydrolysis of the difluoroacetal **2** in aqueous ethanol with Amberlite IR-120 (H^+) resin gave 3,5-dideoxy-3,5-difluoro-D-xylose (**3**) as a chromatographically homogenous, distillable liquid which was characterised as the rather unstable 2,5-dichlorophenylhydrazone.

The three products *A*, *B*, and *C*, formed in addition to the difluoride **2** on treatment of the toluene-*p*-sulphonate **1** with potassium fluoride, were isolated as syrups by column chromatography, in which the sequence of elution was *A*, **2**, *B*, and *C*. Compound *A* (<1%), which had a strong i.r. band at *ca.* 1650 cm^{-1} and

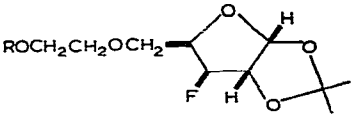
was probably an unsaturated compound, was not investigated further. Compound *C* (*ca.* 5%) was identified by g.l.c. as 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-α-D-xylofuranose (**5**). Compound *B* (*ca.* 9%) was tentatively identified as 3-deoxy-3-fluoro-5-*O*-(2-hydroxyethyl)-1,2-*O*-isopropylidene-α-D-xylofuranose (**4**) on the following evidence. Since compound **4** is an isopropylidene derivative, the ion (221) of highest *m/e* value in the mass spectrum of compound **4** contaminated with compound *C* may be assigned[6] to the $(M-15)^+$ ion of the former compound, indicating a value of 236 for M^+. The $(M-15)^+$ ion for compound *C* gave the expected, intense peak



1 R = TsO
2 R = F
5 R = HO



3



4 R = H
6 R = Ts
7 R = $C_6H_5 \cdot N{=}N \cdot C_6H_4 \cdot CO$

at *m/e* 177. The ^{1}H-n.m.r. spectrum of the chromatographically homogeneous, syrupy toluene-*p*-sulphonate of compound *B* showed signals at τ 2.20–2.80 (4 aromatic protons), 4.20 (doublet, H-1), 4.80 (0.5 proton, low-field portion of H-3), 5.30–6.60 (8–9 protons), and 8.60 and 8.80 (6 protons, isopropylidene Me groups). The ^{19}F spectrum showed one absorption (octet) at ∅ 208.1 p.p.m. upfield with respect to CCl_3F. These data are consistent with structure **6**.

The production of ethers as by-products in attempted displacement of sulphonate groups by fluoride ion in hydroxylic solvents has long been known[7], and the formation of a compound of the type **4**, although apparently not hitherto observed* with ethane-1,2-diol, is not surprising. Contamination of the ethane-1,2-diol with water accounts for the formation of compound *C*, and elimination reactions (compound *A*) are known[3,8] to accompany displacement reactions with fluoride ion.

The biological properties of the difluoride **3** and its utilisation in nucleoside synthesis are being investigated and will be reported elsewhere.

EXPERIMENTAL

Thin-layer chromatography (t.l.c.) was performed on glass plates (3.25 in. sq.) with Kieselgel (Merck, 7731) and detection with iodine vapour or conc. sulphuric acid. Gas–liquid chromatography (g.l.c.) was performed on a Pye Series 104 chromatograph with flame-ionization detection and a 5-ft column of poly(ethylene glycol adipate) at 150°.

*3,5-Dideoxy-3,5-difluoro-1,2-*O*-isopropylidene-α-D-glucofuranose.* — A mixture

*Since this paper was submitted, an unpublished example of the formation of a 2-hydroxyethyl ether has been noted in the synthesis of 6-deoxy-6-fluoro-D-galactose [J. E. G. Barnett, *Advan. Carbohyd. Chem.*, 22 (1967) 179].

of 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-5-*O*-toluene-*p*-sulphonyl-α-D-xylofuranose[3] (6.4 g), anhydrous potassium fluoride (6.7 g, 5 mol), and ethane-1,2-diol (25 ml, distilled from anhydrous potassium carbonate) was boiled under reflux, and the reaction was monitored by t.l.c. (benzene). Reaction was complete in *ca.* 5 min. The cooled mixture was poured into water (50 ml), extracted with chloroform (4 × 50 ml), dried ($MgSO_4$), and concentrated under diminished pressure.

Chromatography of the residue on Kieselgel (Merck, 7734) and elution with benzene yielded the following fractions: I, syrup (30 mg), ν_{max}^{liquid} 1650 cm^{-1} (C=C); II, syrup, which, on distillation, gave 3,5-dideoxy-3,5-difluoro-1,2-*O*-isopropylidene-α-D-xylofuranose (2) (2.2 g, 61%), b.p. 112–114°/~12 mm, $[\alpha]_D^{20}$ −23° (*c* 2, chloroform) (Found: C, 49.8; H, 6.5; F, 19.2. $C_8H_{12}F_2O_3$ calc.: C, 49.5; H, 6.2; F, 19.5%). The compound was homogeneous in t.l.c. and g.l.c. Further elution with benzene–ether (9:1) yielded fraction III which, on distillation, gave a product (0.5 g) having b.p. 120–130°/~0.7 mm.

*3,5-Dideoxy-3,5-difluoro-*D*-xylose.* — A solution of 3,5-dideoxy-3,5-difluoro-1,2-*O*-isopropylidene-α-D-xylofuranose (1 g) in ethanol–water (1:4, 25 ml) containing suspended Amberlite IR-120 (H^+) resin (*ca.* 5 ml) was stirred for 18 h at 60°. The filtered hydrolysate was then concentrated under diminished pressure to yield a crude product which was eluted from Kieselgel (Merck, 7734) with chloroform–ethyl acetate (7:3) to give 3,5-dideoxy-3,5-difluoro-D-xylose (0.36 g), b.p. 115–125° (bath)/0.05 mm, $[\alpha]_D^{22}$ −9° (*c* 1.2, ethanol), which was homogeneous in t.l.c. (R_F 0.4, ethyl acetate–carbon tetrachloride, 1:1) and in paper chromatography (R_G 4.2, organic phase, butyl alcohol–ethanol–water, 4:1:5) (Found: C, 38.8; H, 4.95. $C_5H_8F_2O_3$ calc.: C, 39.0; H, 5.2%).

A solution of the syrupy 3,5-dideoxy-3,5-difluoro-D-xylose (100 mg) and 2,5-dichlorophenylhydrazine (220 mg, 2 mol) in methanol (30 ml) was concentrated on a steam bath. The residue was eluted from Kieselgel (Merck, 7734) with benzene–methanol (9:1) to yield the 2,5-dichlorophenylhydrazone (130 mg, 64%), m.p. 136.5–137° (from aqueous methanol) (Found: C, 42.4; H, 4.1; Cl, 22.3; N, 9.0. $C_{11}H_{12}Cl_2F_2N_2O_2$ calc.: C, 42.2; H, 3.8; Cl, 22.7; N, 9.0%).

Investigation of fraction III. — Examination by g.l.c. revealed two components (*B* and *C*) having relative, integrated peak areas of 2:1. The minor component (*C*) had a retention time identical to that of 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-α-D-xylofuranose[3]. The mass spectrum (MS9 spectrometer, 70 eV, direct injection at 200°) showed, *inter alia*, a peak at 221 (highest *m/e* value) assigned[6] to the $(M-15)^+$ ion of compound *B* [Calc.: $(M-15)^+$ for structure **4**, 221].

Treatment of a portion (130 mg) of fraction III with toluene-*p*-sulphonyl chloride (130 mg) and pyridine (2 ml) for 24 h in the usual manner gave a mixture of sulphonates, of which the minor compound had t.l.c. properties indistinguishable from those of the toluene-*p*-sulphonate **1**. Elution of the mixture from Kieselgel (Merck, 7734) with benzene–ether (95:5) gave the syrupy, chromatographically homogeneous, major component, presumably the toluene-*p*-sulphonate **6**. See Discussion for details of ^{1}H- and ^{19}F-n.m.r. spectra.

Treatment of a portion (130 mg) of fraction III with *p*-phenylazobenzoyl chloride (200 mg) and pyridine (5 ml) for 3 h at 95°, in the usual manner[9], gave a crude product which was eluted from Kieselgel (Merck, 7734) with benzene–ether (95:5) to yield, presumably, 3-deoxy-3-fluoro-1,2-*O*-isopropylidene-5-*O*-(2-*p*-phenylazobenzoyloxyethyl)-α-D-xylofuranose (50 mg), m.p. 76–77° [from light petroleum (b.p. 40–60°)] (Found: N, 6.3; $C_{23}H_{25}FN_2O_6$ calc.: N, 6.3%).

ACKNOWLEDGMENTS

The authors thank Dr. L. F. Thomas and Dr. J. R. Majer (University of Birmingham) for determination of the n.m.r. and mass spectra, respectively. The Sidney Murphy Memorial Fund of the Institute of Cancer Research: Royal Cancer Hospital is acknowledged for a research studentship (to R. H.).

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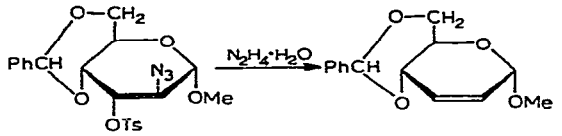
Note

An improved synthesis of methyl 4,6-*O*-benzylidene-α-D-*erythro*-hex-2-enopyranoside

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During the course of investigations (which are still in progress) into the reactions of azido sugars with hydrazine derivatives and hydrazine hydrate (*cf.* ref. 1), a novel and reliable synthesis of methyl 4,6-*O*-benzylidene-α-D-*erythro*-hex-2-enopyranoside (**2**) was discovered. This compound is at present in some demand by a number of workers interested in the migrations of, and additions to, the double bond of unsaturated carbohydrates.

Previous methods for the synthesis of this material necessitated tedious isolation procedures[2]. In our experience, the method[2] based on the procedure of Tipson and Cohen[3] gave variable yields and cannot be satisfactorily employed on a large scale.



1 2

The present method involves the treatment of methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-toluene-*p*-sulphonyl-α-D-altropyranoside (**1**) with excess of hydrazine hydrate, and affords compound **2** in excellent yield, with virtually no isolation procedure; furthermore, the method appears to be applicable on any scale. Although this method involves four steps from methyl 4,6-*O*-benzylidene-2,3-di-*O*-toluene-*p*-sulphonyl-α-D-glucoside, the overall yield is 58–62% (*cf.* 45–55% for the published[2], one-step methods from the same diester). Compound **1** is obtained in good yield from methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-allopyranoside by cleavage of the anhydro ring with sodium azide, followed by toluene-*p*-sulphonylation[4].

EXPERIMENTAL

Methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-toluene-*p*-sulphonyl-α-D-altropyranoside (**1**) (25 g) was heated under reflux with hydrazine hydrate (500 ml), and the reaction was monitored by t.l.c. After 3 h, no starting material remained, and only one new component was present. The colourless solution was cooled, and the

crystals were collected, thoroughly washed with water, and dried to give compound **2** (11.5 g), m.p. 110–112°. On recrystallisation from methanol, compound **2** (11.0 g, 81%) had m.p. 117–118°, $[\alpha]_D^{20}$ +124° (*c* 1.16, chloroform); lit.[5], m.p. 117–119°, $[\alpha]_D^{20}$ +126° (*c* 1.0, chloroform).

The product had i.r., n.m.r., and mass spectra that were identical with those of an authentic sample; a mixed melting point was undepressed.

The above reaction has been investigated with between 0.5 g and 25 g of starting material, and in no case was the yield less than 80%. However, attempts to reduce the proportion of hydrazine hydrate to one half resulted in considerable decrease in the yield.

We thank the Science Research Council for financial support and for the award of a studentship (to G. J. W.).

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Note

The synthesis of D-mannose 6-sulphate

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(Received October 8th, 1968)

D-Mannose 6-sulphate was required for specificity studies on sulphatases from *Patella vulgata*[1], and, although preparations of methyl α-D-mannopyranoside monosulphate[2] and of other undefined mono- and di-sulphates of D-mannose[3] have been reported, a definitive synthesis of the 6-ester had not been previously described.

Sulphation of 1,2,3,4-tetra-*O*-acetyl-β-D-mannopyranose[4] with the pyridine–sulphur trioxide complex[5] in pyridine solution, followed by immediate de-*O*-acetylation with methanolic ammonia, gave crude D-mannose 6-sulphate. Electrophoretic examination indicated that it contained predominantly monosulphate, together with a little D-mannose. Purification of the product by chromatography on a cellulose column, as described[6] for D-glucose sulphates, gave two distinct fractions (Fig. 1).

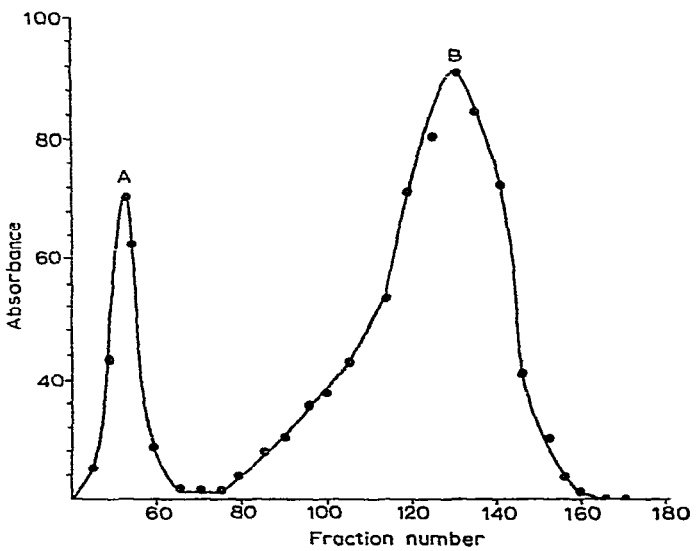


Fig. 1. Purification of D-mannose 6-sulphate on a cellulose column.

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The second fraction (*B*) contained only D-mannose 6-sulphate; this was unresolved by a variety of chromatographic and electrophoretic analyses, and, after isolation in the usual manner, was also analytically pure. The first fraction (*A*) contained D-mannose and D-mannose monosulphate, in approximately equivalent amounts, which may have separated in this manner as the result of complex formation.

EXPERIMENTAL

D-*Mannose 6-(barium sulphate)*. — 1,2,3,4-Tetra-*O*-acetyl-*β*-D-mannose (20.8 g; m.p. 134°; lit.[4], m.p. 135.5–136.5°) in anhydrous pyridine (80 ml) was shaken with pyridine–sulphur trioxide[5] (9.6 g) until dissolution was complete, and the mixture was kept overnight at room temperature. The products were dissolved in water (200 ml) and saturated, aqueous barium hydroxide was added to raise the pH to 7.5. The resulting suspension was concentrated *in vacuo*, and, after addition of water (240 ml), the pH was raised from 3.5 to 7 by the addition of more barium hydroxide. This procedure was repeated four times more, until the product, on addition of water, had pH 7. The insoluble, inorganic residues were removed by centrifugation, and the solution was decolorised with charcoal. On evaporation, a syrup was obtained which crystallised in part when pentyl alcohol was added to its solution in methanol; m.p. 169° (dec.). Recrystallisation caused substantial losses and gave products having variable decomposition points (156–169°). The syrupy product was therefore deacetylated, without purification, by dissolution in dry methanol (260 ml) presaturated at 0° with ammonia, for 12 h. The solution was then evaporated, and the residue, after being washed with alcohol (3 × 400 ml), was dissolved in water (160 ml), and the solution was decolorised with charcoal and concentrated to 15 ml. On addition of methanol (300 ml), the crude product was precipitated, washed with dry ether, and dried over P_2O_5; yield, 11.1 g.

The crude product (6.8 g) in water (12 ml) was applied to a column (80 × 6 cm) of standard-grade cellulose (Whatman) and eluted with 80% aqueous ethanol containing 0.33% formic acid. The fractions (50 ml) were analysed for carbohydrate content[7], appropriately combined (peak *A*, 48–65; peak *B*, 82–160), and concentrated to 12 ml, and methanol (240 ml) and ether (100 ml) were added. The product (4.2 g, 30%) from peak *B*, dried over P_2O_5, had $[\alpha]_D^{20}$ +15.2° (*c* 0.5, water) [Found: Ba, 20.9; S, 9.6. $(C_6H_{11}O_9S)_2Ba$ calc.: Ba, 20.9; S, 9.8%].

ACKNOWLEDGMENTS

We thank Professor S. Peat, F. R. S., for his interest in this work, and the Science Research Council for financial assistance.

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Carbohyd. Res., 10 (1969) 174–176

Preliminary communication

Determination of bond-types of polysaccharides

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Two different methods are generally applied in the determination of the bond-type of polysaccharides: periodate oxidation[1-4] and methylation followed by hydrolysis[5] The second method gives very accurate results but is rather tedious. We now report a development of the periodate-oxidation approach.

The polyalcohol obtained in the first stage of the Smith degradation of a poly-

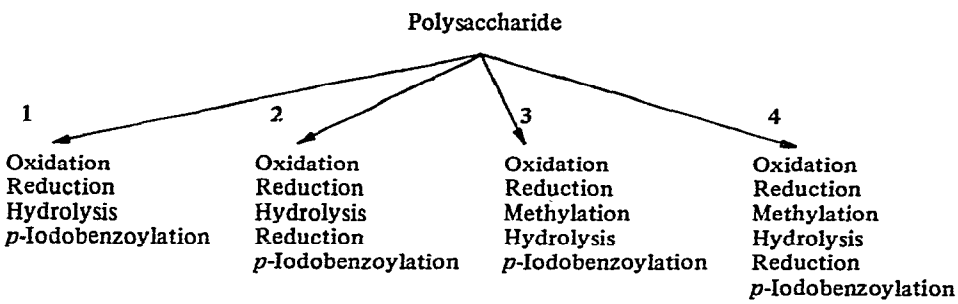


Fig.1. Degradation schemes for a polysaccharide.

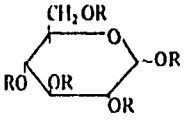
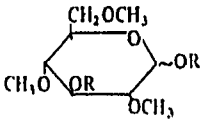
TABLE I

DATA ON *p*-IODOBENZOATES[a]

Compound	*Melting point (degrees)*	$[\alpha]_D^{22}$ *(chloroform) (degrees)*	*T.l.c.* (R_F) A^b	B^b
1,2-Di(*p*-iodobenzoyloxy)ethane	146–147	–	0.38	0.60
Tri-*O*-(*p*-iodobenzoyl)glycerol	171–173	–	0.32	0.66
Tetra-*O*-(*p*-iodobenzoyl)erythritol	225–226	–	0.43	0.71
Hexa-*O*-(*p*-iodobenzoyl)-D-glucitol	115–117	+42.4 (*c* 0.5)	0.24	0.78
p-Iodobenzoylglycoaldehyde	250–251	–	0.15	0.38
Di-*O*-(*p*-iodobenzoyl)-D-glyceraldehyde	156–158	~0	0.18	0.35
Penta-*O*-(*p*-iodobenzoyl)-α-D-glucopyranose	244–246	+155 (*c* 0.2)	0.23	0.50
Penta-*O*-(*p*-iodobenzoyl)-β-D-glucopyranose	279–280	−5.3 (*c* 0.2)	0.20	0.46

[a]Satisfactory elemental analyses were obtained for all of the esters.
[b]*A*, benzene; *B*, benzene–methanol (99:1). An excellent separation could be obtained by using solvents *A* and *B* successively in two-dimensional t.l.c.

TABLE II
DEGRADATION FRAGMENTS CHARACTERISTIC OF VARIOUS BOND TYPES

Bond types	D-*Glucopyranose, methyl ethers*	*Degradation fragments*[a]			
		1	2	3	4
D-*Gp*-(1→	2,3,4,6-tetra-	CH_2OR $CHOR$ CH_2OR CHO CH_2OR $HCOOH$	CH_2OR $CHOR$ CH_2OR CH_2OR $HCOOH$	CH_2OCH_3 $CHOR$ CH_2OCH_3 CHO CH_2OCH_3 $HCOOH$	CH_2OCH_3 $CHOR$ CH_2OCH_3 CH_2OR CH_2OCH_3 $HCOOH$
→2)-D-*Gp*-(1→	3,4,6-tri-	CH_2OR $CHOR$ CH_2OR CHO $HCOR$ CH_2OR	2 CH_2OR $CHOR$ CH_2OR	CH_2OCH_3 $CHOR$ CH_2OCH_3 CHO $HCOR$ CH_2OCH_3	CH_2OCH_3 $CHOR$ CH_2OCH_3 CH_2OR $CHOR$ CH_2OCH_3
→3)-D-*Gp*-(1→	2,4,6-tri-		CH_2OR $HCOR$ $ROCH$ $HCOR$ $HCOR$ CH_2OR		CH_2OR $HCOCH_3$ $ROCH$ $HCOCH_3$ $HCOR$ CH_2OCH_3
→4)-D-*Gp*-(1→	2,3,6-tri-	CH_2OR $HCOR$ $HCOR$ CH_2OR CHO CH_2OR	CH_2OR $HCOR$ $HCOR$ CH_2OR CH_2OR	CH_2OCH_3 $HCOR$ $HCOR$ CH_2OCH_3 CHO CH_2OCH_3	CH_2OCH_3 $HCOR$ $HCOR$ CH_2OCH_3 CH_2OR CH_2OCH_3
→6)-D-*Gp*-(1→	2,3,4-tri-	CH_2OR $CHOR$ CH_2OR CHO CH_2OR $HCOOH$	CH_2OR $CHOR$ CH_2OR CH_2OR $HCOOH$	CH_2OCH_3 $CHOR$ CH_2OR CHO CH_2OCH_3 $HCOOH$	CH_2OCH_3 $CHOR$ CH_2OR CH_2OR CH_2OCH_3 $HCOOH$
→2)$_n$ →6)-D-*Gp*-(1→	3,4-di-	CH_2OR $CHOR$ CH_2OR CHO $HCOR$ CH_2OR	2 CH_2OR $CHOR$ CH_2OR	CH_2OCH_3 $CHOR$ CH_2OR CHO $HCOR$ CH_2OCH_3	2 CH_2OR $CHOR$ CH_2OCH_3

→3)-[D-Glcp-(1→ →6)]-D-Glcp-(1→	2,4-di-		CH2OR HCOR ROCH HCOR HCOR CH2OR		CH2OR HCOCH3 ROCH HCOCH3 HCOR CH2OR
→4)-D-Glcp-(1→ →6)-D-Glcp-(1→	2,3-di-	CH2OR HCOR HCOR CH2OR	CH2OCH3 HCOR HCOR CH2OR	CH2OCH3 CHO CH2OCH3	CH2OCH3 HCOR HCOR CH2OR
→2)-D-Glcp-(1→ →3)-D-Glcp-(1→	4,6-di-		CH2OR HCOR ROCH HCOR HCOR CH2OR		CH2OR HCOR ROCH HCOCH3 HCOR CH2OCH3
→2)-D-Glcp-(1→ →4)-D-Glcp-(1→	3,6-di-		CH2OR HCOR ROCH HCOR HCOR CH2OR		CH2OR HCOR CH3OCH HCOR HCOR CH2OCH3
→3)-D-Glcp-(1→ →4)-D-Glcp-(1→	2,6-di-		CH2OR HCOR ROCH HCOR HCOR CH2OR		CH2OR HCOCH3 ROCH HCOR HCOR CH2OCH3

[a] R = *p*-iodobenzoyl.

saccharide can easily be methylated, and this approach has been used to clarify the fine structure of amylopectin[6], cellulose[7], and amylose[8]. However, the Smith degradation has not been applied for the complete determination of the structure of polysaccharides. Several variations of the Smith degradation have now been evolved, in order to obtain fragments that are characteristic of all bond-types, and these are shown in Fig. 1, together with the reaction sequence (1) involved in the standard Smith-degradation. It will be seen from Fig. 1 that the final stage in each sequence involves *p*-iodobenzoylation. The *p*-iodobenzoates are readily isolated by crystallization (Table I), and if ^{131}I is incorporated into the reagent, identification of the esters may be effected by the reversed, isotopic-dilution method. Alternatively, after separation by t.l.c., the esters can be located and identified by the radio-activity on the chromatoplate. The above methods of fragment identification are comparable in efficiency with that of the classical methylation approach.

The degradation sequences (1–4, Fig. 1) permit the identification of all bond-types, and the procedure is illustrated in Table II, in terms of glucans variously containing eleven bond-types. Glucose residues that are resistant to periodate oxidation will be finally transformed into a mixture of α- and β-penta-*p*-iodobenzoates which may be difficult to resolve. In this case, it is expedient to introduce a second reduction[10] stage, using sodium borohydride, after hydrolysis of the reduced and methylated polysaccharide (Fig. 1, 4), followed by conversion of the glucitol derivatives into the *p*-iodobenzoates.

Fragment identification by the above method involving radioactive *p*-iodobenzoates can be performed successfully on 10^{-8}–10^{-9} gmole of any one fragment.

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Preliminary communication

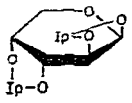
Acid-catalyzed hydrolysis of isopropylidene acetals of some 2-pentuloses and 2-hexuloses

R. STUART TIPSON, BARBARA F. WEST, and ROBERT F. BRADY, JR.

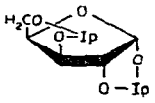
Analytical Chemistry Division, Institute for Materials Research, National Bureau of Standards, Washington, D.C. 20234 (U. S. A.)

(Received January 28th, 1969)

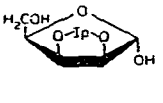
For acid-catalyzed hydrolysis of derivatives of ketoses, the use of such hydrolysts as hydrochloric acid or sulfuric acid leads to considerable decomposition of the free ketose liberated. This difficulty was overcome by use, as the hydrolyst, of oxalic acid[1]. In an earlier study[2], it was found that 100 mM oxalic acid is suitable for effecting, and in determining the rate of, hydrolysis of a variety of isopropylidene acetals of pentoses at 65°.



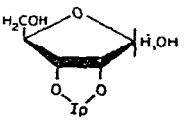
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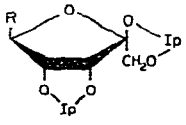
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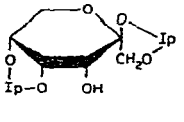
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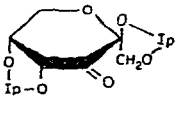
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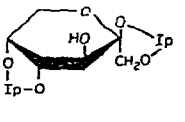
5a R = H
5b R = CH_2OH



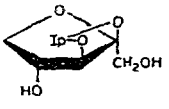
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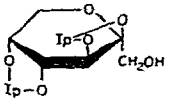
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9

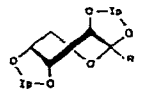


10

It has previously been shown[2] that, under these standard conditions of hydrolysis, the per-isopropylidenated aldopentoses [namely, 1,2:3,4-di-*O*-isopropylidene-β-L-arabino-pyranose (1-e, the enantiomorph of 1), 1,2:3,5-di-*O*-isopropylidene-α-D-xylofuranose (2), 2,3-*O*-isopropylidene-α-D-lyxofuranose★ (3), and 2,3-*O*-isopropylidene-D-ribofuranose (4)] are completely hydrolyzed to the corresponding free sugar within 2 h at 65°,

★The furanoid structure and anomeric configuration of this compound, first crystallized and reported by Levene and Tipson[2], have since been proved[3].

regardless of (*a*) the presence of a pyranoid (**1**) or a furanoid ring (**2**–**4**), or (*b*) the positions of attachment of the isopropylidene groups. However, as previously noted[2], hydrolysis of **2** to 1,2-*O*-isopropylidene-α-D-xylofuranose is complete in 40 min at 25°, but hydrolysis of **1**-**e** to a monoisopropylidene acetal, at either 25° or 65°, could not be detected polarimetrically. We have now studied the hydrolysis of **1**.



1 R = H
10 R = CH_2OH

Complete hydrolysis was effected in 1.5 h at 65°, but the presence of any compound besides **1** and D-arabinose in the reaction mixture at any time could not be detected by t.l.c.★. Hence, as regards acetals **1**–**4**, the *m*-dioxane ring *cis*-fused at C-3,4 of **2** is much less stable than any of the *cis*-fused 1,3-dioxolane rings.

In contrast to these results for the acetals (**1**–**4**) of the aldopentoses, the sole 2-pentulose acetal for which a detailed study was made, namely, 2,3-*O*-isopropylidene-β-D-*threo*-pentulofuranose† (**9**), required 8 h for complete hydrolysis. We have therefore determined, by the technique described earlier[2], the rate of hydrolysis of the diacetal of the other D-2-pentulose, namely, 1,2:3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose (depicted as the β-D anomer **5a**), and have compared it with the rates for some D-2-hexulose acetals [1,2:4,5-di-*O*-isopropylidene-β-D-*ribo*-hexulopyranose†† (**6**), 1,2:4,5-di-*O*-isopropylidene-β-D-*erythro*-2,3-hexodiulo-2,6-pyranose†† (**7**), 1,2:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexulopyranose†† (**8**), and 2,3:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexulopyranose (**10**)]. The results★★ are given in Fig.1, from which it may be seen that these six compounds fall into three groups: **5a** (~ 1.25 h); **6**, **7**, and **8** (2–3 h); and **9** and **10** (8 h). Thus, of the four acetals (**5a**–**8**) having a *spiro* structure involving C-1 and C-2, the furanoid compound **5a** is hydrolyzed about twice as fast as the pyranoid compounds (**6**, **7**, and **8**).

Presence of two *spiro*-fused, 5-membered rings in compound **5a** evidently induces instability in the 1,2-isopropylidene group, because graded hydrolysis of **5a** gives a monoacetal that we have isolated, and characterized as 3,4-*O*-isopropylidene-D-*erythro*-pentulofuranose, whereas graded hydrolysis of **6** (see ref. 6) and **8** (see ref. 8), in which there is less strain at C-2, causes removal of the 4,5-isopropylidene group. In contrast, the acetals **9** and **10** (that lack the *spiro* structure but have a hydroxymethyl group attached to C-2) are completely hydrolyzed at about one quarter of the rate found for compounds **6**, **7**, and **8**, regardless of the presence of a furanoid (**9**) or a pyranoid ring (**10**)‡. This behavior must be attributable to a stabilizing effect of the hydroxymethyl group at C-2 (compare **9** with **2**, and **10** with **1**).

★On Kieselguhr G (preheated for 1 h at 110°) with 2:1 (v/v) ethyl acetate–pentane (detection with sulfuric acid spray, and charring).

†This complete structure was later proved[4].

††The β-D anomeric configuration of compounds **6** and **7** is assigned because of their preparation[5,6] from compound **8**, to which this configuration had been assigned by Ohle and Just[7].

★★The rates of hydrolysis of compounds **8** and **10** in 0.1% hydrochloric acid at 80° had previously been compared[8]. Their rates in 100 mM oxalic acid at 65° are now given.

‡Graded hydrolysis of **10** with acid[11] causes removal of the 4,5-isopropylidene group, to give a pyranoid structure resembling the furanoid structure **9**.

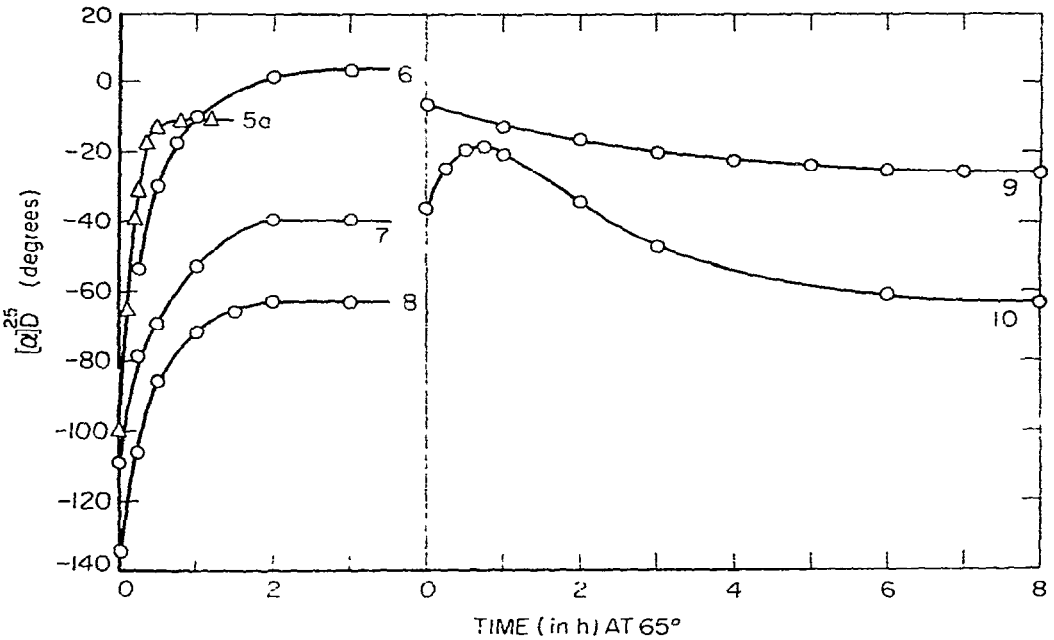


Fig.1. Rate of hydrolysis of 1,2:3,4-di-*O*-isopropylidene-D-*erythro*-pentulose (**5a**), 1,2:4,5-di-*O*-isopropylidene-β-D-*ribo*-hexulopyranose (**6**), 1,2:4,5-di-*O*-isopropylidene-β-D-*erythro*-2,3-hexodiulo-2,6-pyranose (**7**), 1,2:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexulopyranose (**8**), 2,3-*O*-isopropylidene-β-D-*threo*-pentulofuranose (**9**), and 2,3:4,5-di-*O*-isopropylidene-β-D-*arabino*-hexulopyranose (**10**) in 100 mM oxalic acid (aqueous) at 65°. All values for $[\alpha]_D$ are calculated on the original weight of acetal. The final specific rotations, recalculated as the respective free sugar, are: for **5a**, -16.6° (lit.[2] for enantiomorph, +16.6°); **6**, +4.7° (lit.[9] +4.7°); **7**, -43.8°; **8**, -91.2° (lit.[10] -92.0°); **9**, -33.2° (lit.[2] -33.2°); and **10**, -90.3° (lit.[10] -92.0°).

Determination of the rate of acid-catalyzed, complete hydrolysis to the free sugar, under the conditions described, therefore appears promising as a simple, rapid method for determining (*a*) the presence or absence of a *spiro* structure involving C-1 and C-2 in cyclic acetals of 2-ketoses (fast or slow hydrolysis, respectively), and (*b*) the presence of a furanoid ring if this *spiro* structure is present (very fast hydrolysis). As a check, hydrolysis of 1,2:3,4-di-*O*-isopropylidene-β-D-*ribo*-hexulofuranose (**5b**) was studied; it was rapid (~ 50 min for completion).

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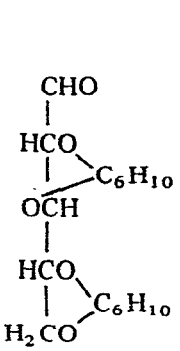
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Preliminary communication

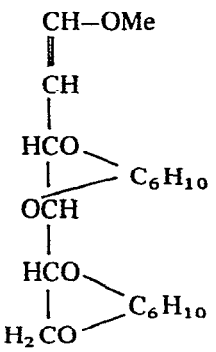
A novel synthesis of 2-deoxyhexoses

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(Received January 6th, 1969; in revised form, February 3rd, 1969)

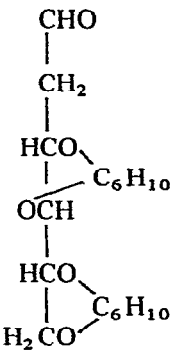
The recently reported[1,2] synthesis of carbohydrate vinyl ethers by a Wittig coupling of *aldehydo* sugars with alkoxymethylenephosphoranes affords a route to 2-deoxy sugars.



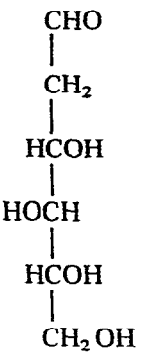
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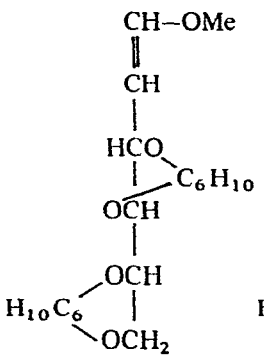
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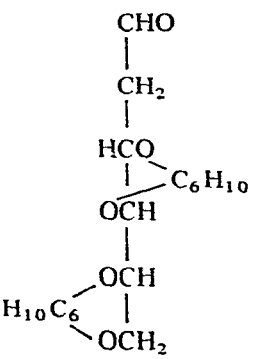
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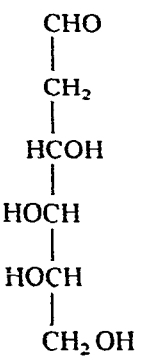
4



5



6



7

Thus, condensation of 2,3:4,5-di-*O*-cyclohexylidene-*aldehydo*-D-xylose (**1**) with methoxymethylenephosphorane in dry ether under nitrogen at –70° afforded 2-deoxy-

3,4:5,6-di-*O*-cyclohexylidene-1-*O*-methyl-D-*xylo*-hex-1-enitol (2) as a colourless syrup (32%), $[\alpha]_D^{27}$ −44° (*c* 3.4, chloroform), R_F 0.75 (t.l.c., alumina, benzene), ν_{max}^{liquid} 1672 cm^{-1} (vinyl ether[3]) (Found: C, 68.1; H, 9.1. $C_{19}H_{30}O_5$ calc.: C, 67.45, H, 8.9%).

In a similar manner, 2,3:4,5-di-*O*-cyclohexylidene-*aldehydo*-L-arabinose was converted into 2-deoxy-3,4:5,6-di-*O*-cyclohexylidene-1-*O*-methyl-L-*arabino*-hex-1-enitol (5), isolated as a colourless syrup (21%), $[\alpha]_D^{20}$ −18° (*c* 2.6, chloroform), R_F 0.7, ν_{max}^{liquid} 1675 cm^{-1} (Found: C, 68.0; H, 9.1%).

The vinyl ethers 2 and 5 rapidly decolourized a solution of bromine in carbon tetrachloride, and, on brief treatment with ether saturated with 72% perchloric acid, were converted, respectively, into the aldehydes 2-deoxy-3,4:5,6-di-*O*-cyclohexylidene-D-*xylo*-hexose (3), colourless syrup (86%), $[\alpha]_D^{20}$ −15° (*c* 2.3, chloroform), R_F 0.2 (t.l.c., alumina, chloroform); ν_{max}^{liquid} 1734 cm^{-1} (C=O) (Found: C, 64.1; H, 8.9. $C_{18}H_{28}O_5$ calc.: C, 63.6; H, 8.6%); and 2-deoxy-3,4:5,6-di-*O*-cyclohexylidene-L-*arabino*-hexose (6), colourless syrup (88%), $[\alpha]_D^{22}$ +16° (*c* 1.25, chloroform), R_F 0.25, ν_{max}^{liquid} 1740 cm^{-1} (Found: C, 64.2; H, 8.8%).

Treatment of the acetal 3 with 50% aqueous acetic acid gave 2-deoxy-D-*xylo*-hexose (4, 70%), $[\alpha]_D^{22}$ +15° (*c* 1.8, water), lit.[4] $[\alpha]_D^{22}$ +12 ±2° (water), characterised as the *p*-toluidine derivative, m.p. 139°, $[\alpha]_D^{20}$ −63° (*c* 2.1, ethanol); lit.[4], m.p. 141–142°, $[\alpha]_D^{22}$ −66.9° (ethanol). Likewise, the acetal 6 was converted into 2-deoxy-L-*arabino*-hexose (7, 74%), $[\alpha]_D^{20}$ +41° (*c* 2, water) (Found: C, 43.4; H, 7.5. $C_6H_{12}O_5$ calc.: C, 43.9; H, 7.3%) which was characterized as the aniline derivative, m.p. 176–178°, $[\alpha]_D^{21}$ −34° (*c* 2, ethanol) (Found: C, 60.3; H, 7.6; N, 6.0. $C_{12}H_{18}NO_4$ calc.: C, 60.0; H, 7.5; N, 5.8%).

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Preliminary communication

The periodate-oxidation limit of alginate

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(Received February 3rd, 1969)

Despite strong evidence[1-3] that they are entirely (1→4)-linked, alginates do not, under conditions that avoid overoxidation, consume more than 0.45–0.55 mol. of periodate per "anhydrohexuronic acid" residue[4,5]. The possibility[2,4,5] that hydroxyl groups are blocked by lactonisation, intermolecular esterification, or other forms of *O*-substitution must be rejected on the basis of much experience in this and other[2] laboratories. Evidence is now reported that the abnormal oxidation-limit is caused by intramolecular hemiacetal formation[6].

When sodium alginate was oxidised in 0.01 M periodate to its limiting consumption of 0.45 mol. of oxidant, and then reduced with sodium borohydride, it again became susceptible to periodate, and further oxidation proceeded smoothly up to a second oxidation-limit, corresponding to a total consumption of 0.95 mol. of oxidant per "anhydrohexuronic acid" unit. A second reduction with borohydride then permitted a small further uptake of periodate, bringing the total consumption close to the theoretical limit of 1.0 mol. Pretreatment of the alginate with borohydride prior to oxidation did not change the first oxidation-limit.

Throughout this sequence of reactions, the material gave no reaction with Schiff's reagent, its neutralisation equivalent (as determined by titration with cetylpyridinium chloride[7]) remained at the theoretical value for intact alginate (*ca.* 200), and its molecular weight remained high (>20,000) as indicated by its total exclusion from Sephadex G-50. The changes in specific rotation were especially significant, and are indicated in Table I.

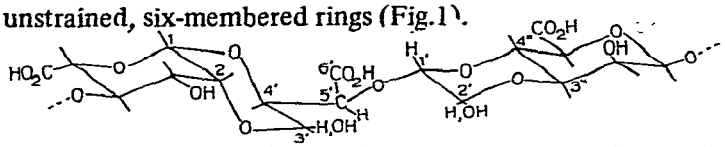
TABLE I

CHANGES IN THE SPECIFIC ROTATION OF *LAMINARIA DIGITATA* ALGINATE DURING SEQUENTIAL PERIODATE-OXIDATION AND REDUCTION

Material	*Identifying letter*	$[\alpha]_D^{22}$ *(degrees)*
Sodium alginate	*A*	−88
Limit-oxidised *A*	*B*	+102
Reduced *B*	*C*	−36
Limit-oxidised *C*	*D*	+113
Reduced *D*	*E*	−7
Limit-oxidised *E*	*F*	−2

The first oxidation-limit was essentially independent of the concentration of alginate and of the presence of other polysaccharides simultaneously oxidised in the same solution. Anomalous oxidation-limits were also shown by cellulose and amylose, partially oxidised at C-6 with nitrous acid. An oxycellulose containing 65.3% of D-glucuronic acid showed a first oxidation-limit of 0.44 mol. of periodate, and for an oxyamylose containing 30.3% of D-glucuronic acid, the first limit was 0.76 mol. Both materials still contained unoxidised D-glucuronic acid and D-glucose residues at these limits.

If the aldehyde groups of oxidised residues formed hemiacetals with the hydroxyl groups of unoxidised residues that were situated at remote positions in the same polymer molecule, limit-oxidised alginate would be a very compact and rigid molecule. In fact, its viscosity in solution is highly dependent upon the concentration of added salt, showing that it is flexible and expanded in dilute solutions[8]. On the other hand, molecular model-building indicates that hemiacetal formation between nearest neighbours could give rise to unstrained, six-membered rings (Fig. 1).



On the assumption that oxidation proceeds initially in a random manner, that only one residue in the chain suffers oxidation at any instant in time, and that its two nearest neighbours are then instantaneously protected from future oxidation, it was calculated that the first oxidation-limit would be 0.48 mol. Possible reasons for variation in the observed limit about this calculated value will be discussed elsewhere. The stated assumptions also predict a second oxidation-limit of 0.95 mol., because they require that, at the first limit, the molecule should contain not only alternating sequences of oxidised and unoxidised residues, but also groups of two consecutive, unoxidised residues. Further oxidation after reduction would then lead to attack of all of the isolated, unoxidised residues, but of only one residue in each of the unoxidised doublets.

If this reaction scheme is correct, Barry degradation[9] of alginate, after oxidation to its first limit, should yield the phenylosazones of mono- and di-uronides in a molar ratio of approximately 10:1, but not the phenylosazone of any larger oligouronide. Such a result was in fact obtained.

Inter-residue, intramolecular, hemiacetal formation of the kind indicated by the present evidence was recently postulated to explain the anomalous periodate-oxidation of a bacterial polysaccharide containing residues of mannuronic acid[10]. Further work is in progress in an attempt to clarify the role of the carboxyl group in promoting this reaction.

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Book review

Progress in Phytochemistry (Volume 1): edited by L. REINHOLD and Y. LIWSCHITZ, The Hebrew University of Jerusalem, Israel. Interscience Publishers, 1968, 723 pp., £9.

The plant kingdom has long been the happy hunting-ground of the organic chemist who has characterised a multitude of constituents, many of them having fascinating structures. Many plant products find important applications in medicine, and the search continues for new natural products of novel structure and biological action. Modern techniques have considerably facilitated their separation and identification; consequently, the chemistry of plant products is progressing at a great pace. Furthermore, there is now considerable interest in the biosynthesis of plant constituents, coupled with studies of their metabolism and function, and the control mechanisms for these processes. There is also the important use of chemistry in plant classification.

The appearance of the first volume of this new series is well overdue, in view of the general lack of authoritative accounts of developments in the phytochemical field. The initiative of the editors and their advisory board in launching this new series, at a time when the general climate to any new venture of this kind is unfavourable, is to be commended. It represents a major contribution to the advancement of the subject, a relatively new, but rapidly expanding, field of study that impinges upon large areas of the biochemical, chemical, and plant sciences.

Eleven topics are included, and they are: (1) acyl lipids and fatty acids of photosynthetic tissue, (2) chemistry of plant cuticles, (3) relationship between plant-growth hormones and nucleic acid metabolism, (4) recent advances in the chemistry of the tetracyclic diterpenes, (5) biosynthesis of the gibberellins, (6) lichen substances, (7) biochemistry and physiology of phytochrome, (8) biochemistry and postulated mechanisms of nitrogen fixation, (9) the tropane alkaloids, (10) biochemical systematics, and (11) constituents of ferns.

The contributors are well-known, international authorities in the respective fields, and, in accordance with the stated editorial policy, they have included sufficient background material for the non-specialist to get to grips quickly with their critical appraisal of the subject. All research workers in the phytochemical field will want access to this volume, and it is recommended, without reservation, to libraries of all chemical, biochemical, and plant-science departments. There is much in it to interest carbohydrate chemists, and the book will also be required by teachers and advanced students who wish to keep abreast of current problems and recent developments in phytochemical topics. The production is excellent, each chapter has an extensive bibliography in which the literature is covered well into 1967, and there is a good subject index.

If the standard of this volume is maintained in future volumes, this series will undoubtedly establish itself as the standard reference work for topics in phytochemistry.

L. HOUGH (*London*)

Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

SYNTHESIS OF 2-AMINO-3-*O*-(D-1-CARBOXYETHYL)-2-DEOXY-D-GALACTOSE (D-*galacto* ANALOG OF MURAMIC ACID) AND OF ITS (L-1-CARBOXYETHYL) ANALOG*

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(Received October 28th, 1968)

ABSTRACT

The synthesis of syrupy 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-galactose ("galactomuramic acid") and of crystalline 2-amino-3-*O*-(L-1-carboxyethyl)-2-deoxy-D-galactose ("galactoisomuramic acid"), starting from benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside, is described.

INTRODUCTION

A new disaccharide has been isolated recently from the lysozyme digest of *Micrococcus lysodeikticus* cell-wall. It was shown to be composed of a 2-amino-2-deoxy-D-glucose residue and of a new amino sugar similar to, but not identical with, 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (muramic acid). Degradation of this sugar indicated a structure having the D-*manno* configuration[1,2]. In order to study the separation by paper, thin-layer, and gas–liquid chromatography of compounds having compositions similar to that of muramic acid, the D-*galacto* analogs of both muramic acid and isomuramic acid [2-amino-3-*O*-(L-1-carboxyethyl)-2-deoxy-D-glucose] have been synthesized.

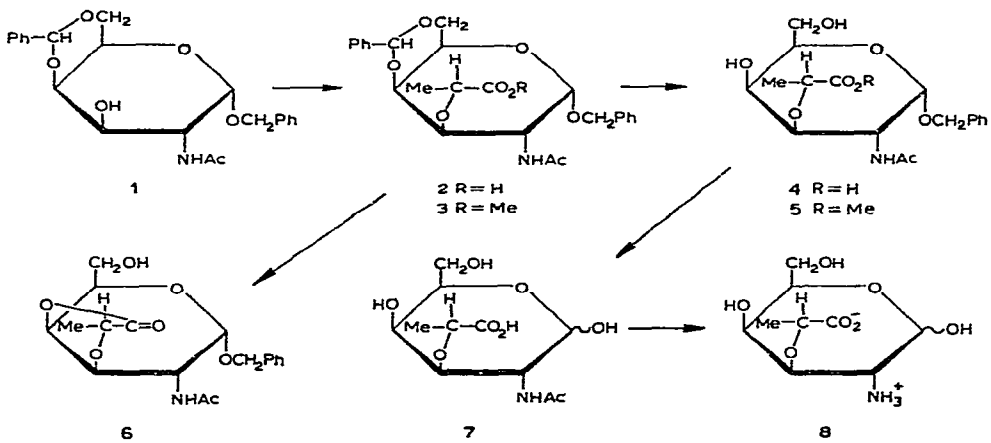
DISCUSSION

The synthesis, similar to that recently described for the preparation of muramic acid[3], was started from benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside[4] (1), with the exception that pure L-2-chloropropionic acid and D-2-

*Amino Sugars LVI. This is publication No. 469 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by research grants from the National Institute of Allergy and Immunology, National Institutes of Health, United States Public Health Service (Grant AI-06692), and from the National Science Foundation (Grant GB-5031). A preliminary communication has been presented [*Abstracts Papers Amer. Chem. Soc. Meeting*, San Francisco, 155 (1968) C-013].

**Fellow of the Centre National de la Recherche Scientifique (France).

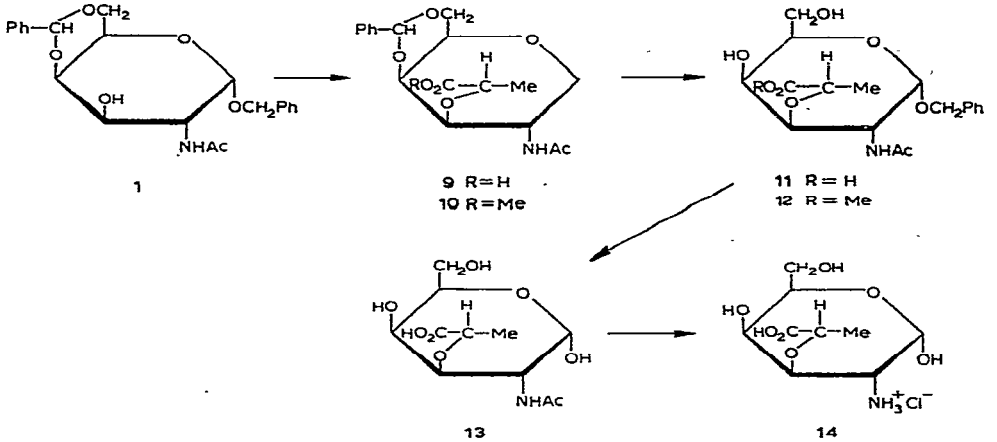
chloropropionic acid, instead of DL-2-chloropropionic acid, were used. Both acids were prepared by a modification[5] of the method of Fischer and Raske[6]. After condensation of **1** with L-2-chloropropionic acid, benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**2**) was isolated in 80% yield. Esterification of **2** with diazomethane gave the crystalline ester **3**, whereas removal of the benzylidene group of **2** with acetic acid transformed **2** into crystalline benzyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**4**). An attempt to obtain the methyl ester **5** from **4** by treatment with diazomethane was, surprisingly, unsuccessful. An attempt to obtain the methyl ester **5** from **3** by treatment with dilute acetic acid was also disappointing, since the methyl ester group was partially hydrolyzed to give **4**, with the concomitant formation of a small proportion of internal ester **6**.



Hydrogenolysis of the protective benzyl glycoside group of **4** gave amorphous 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-galactose ("*N*-acetylgalactomuramic acid") (**7**), which was further hydrolyzed to afford amorphous 2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-galactose ("galactomuramic acid") (**8**).

A similar sequence of reactions starting with D-2-chloropropionic acid gave the 3-*O*-(L-1-carboxyethyl) derivatives **9** to **14**. It is of interest that, in this case, treatment of benzyl 2-acetamido-3-*O*-(L-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**11**) with diazomethane gave the methyl ester **12** in excellent yield, in contrast to the results obtained with the 3-*O*-(D-1-carboxyethyl) derivative **4**. This difference in reactivity of the carboxylic group toward diazomethane might be explained by a change of the pK due to hydrogen bonding[7]. The configurational factors are, however, not clear at the present time, since the 3-*O*-(D-1-carboxyethyl) derivative having the D-*gluco* configuration is reactive[8], but those having D-*galacto* and D-*manno*[9] configurations are not, whereas the 3-*O*-(L-1-carboxyethyl) derivative having the D-*galacto* configuration is reactive, but not that having the D-*gluco* configuration[10]. Hydrogenolysis of the benzyl group of **11** gave crystalline 2-acetamido-3-*O*-(L-1-carboxyethyl)-2-deoxy-α-D-

galactose ("*N*-acetylisogalactomuramic acid") (**13**), which was further hydrolyzed into 2-amino-3-*O*-(L-1-carboxyethyl)-2-deoxy-D-galactose ("isogalactomuramic acid"), isolated as the crystalline hydrochloride **14**.



In the attribution of the D configuration to the lactyl residue linked to O-3 of the 2-amino-2-deoxy-D-glucose moiety in muramic acid, it was assumed[11] that a D-lactyl side-chain would give a more dextrorotatory contribution to the molecule than does an L-lactyl side-chain. This assumption is not always correct, as shown by a comparison of the optical rotation of derivatives **2** and **9** in pyridine solution: the 3-*O*-(L-carboxyethyl) derivative **9** is more dextrorotatory ($[\alpha]_D$ +223°) than the 3-*O*-(D-carboxyethyl) derivative **2** ($[\alpha]_D$ +174°). This result shows the necessity for more unequivocal evidence regarding the configuration of this part of the muramic acid molecule.

EXPERIMENTAL

Melting points were determined on a hot stage equipped with a microscope, and correspond to "corrected melting points". Optical rotations were determined with a Perkin–Elmer No. 141 polarimeter. The chloroform used was A.R. grade and contained approximately 0.75% of ethanol. I.r. spectra were recorded, for potassium bromide discs, with a Perkin–Elmer spectrophotometer Model 237. N.m.r. spectra were recorded with a Varian T-60 n.m.r. Spectrometer. The homogeneity of the compounds synthesized was determined by chromatography on plates covered with a thin layer of a 3:1 mixture of silica gel G (Merck) and silica gel GF (Merck). Column chromatography was performed on "Silica Gel Davison", from the Davison Co., Baltimore, Maryland 21201 (grade 950, 60–200 mesh), which was used without pretreatment. When deactivation by contact with moist air occurred, reactivation was conducted by heating to 170–200° (manufacturer's instructions). The sequence of eluants was hexane, benzene (or 1,2-dichloroethane), ether, ethyl acetate, acetone, and methanol, individually or in binary mixtures. The ratio of weight of substance

to weight of adsorbent was 1:50 to 1:100. The ratio of weight of substance (in g) to volume of fraction of eluant (in ml) was 1:100. The ratio of diameter to length of the column was 1:20. Evaporations were conducted *in vacuo*, with the bath temperature below 45°. Volumes of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were performed by Dr. M. Manser, Zurich, Switzerland.

Benzyl 2-acetamido-4,6-O-benzylidene-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**2**). — To a stirred solution of dry benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside[3] (**1**) (0.44 g) in dry *p*-dioxane (30 ml), freshly distilled in the presence of sodium, was added sodium hydride in oil suspension (0.25 g) (50% of sodium hydride by weight, Alfa Inorganics, Beverly, Massachusetts). The mixture was kept for 1 h at 95°, and then the temperature was decreased to 65°, and L-2-chloropropionic acid (0.62 g) dissolved in a small volume of dry *p*-dioxane was added. L-2-Chloropropionic acid was prepared by a modification[5] of the method of Fischer and Raske[6] for D-2-bromopropionic acid. After 1 h, a further addition of the 50% suspension of sodium hydride (1 g) was made, and the stirring was continued overnight at 65°. To the cooled reaction mixture, water (15 ml) was carefully added, to decompose the excess of sodium hydride. The lower layer, alkaline and dark-colored, was discarded, and the upper, organic layer was decanted, filtered, partially concentrated (about 30 ml was evaporated off), and diluted with 10 ml of water. This aqueous solution was extracted once with chloroform, to remove the mineral oil and any trace of unreacted starting material; it was then acidified at 0° with 2.5M hydrochloric acid until pH 3 was reached. The resulting precipitate was immediately extracted with chloroform. The chloroform extracts were combined, washed with water, and evaporated, to give a crystalline residue (0.42 g, 80%) of a product that was practically pure, as shown by t.l.c. in 2:1 acetone–methanol; after recrystallization from ethyl acetate, it had m.p. 219–220°, $[\alpha]_D^{20}$ +174° (*c* 0.32, pyridine), $[\alpha]_D^{20}$ +188° (*c* 0.40, methanol).

Anal. Calc. for $C_{25}H_{29}NO_8$: C, 63.68; H, 6.20; N, 2.97. Found: C, 63.73; H, 6.26; N, 3.03.

Benzyl 2-acetamido-4,6-O-benzylidene-3-O-[D-1-(methoxycarbonyl)ethyl]-2-deoxy-α-D-galactopyranoside (**3**). — The crude, crystalline product **2** (0.42 g) was dissolved in methanol (25 ml), and esterified by addition of a slight excess of diazomethane in ether. The course of the reaction was followed by t.l.c. in 2:1 acetone–methanol. Evaporation of the solution, and crystallization of the residue from ethyl acetate, gave 254 mg (60%), m.p. 198–200°, $[\alpha]_D^{20}$ +180° (*c* 1.75, chloroform). Slow crystallization from methanol gave long needles, m.p. 201.5–203.5°.

Anal. Calc. for $C_{26}H_{31}NO_8$: C, 64.32; H, 6.44; N, 2.88; O, 26.36. Found: C, 64.24; H, 6.50; N, 3.03; O, 26.62.

Benzyl 2-acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**4**). —A solution of compound **2** (242 mg) in 60% acetic acid (3.2 ml) was heated for 1 h on a boiling water-bath. After being cooled to room temperature, the solution was evaporated to a glassy residue, and the last traces of acetic acid and benzaldehyde were

removed by adding and evaporating several portions of water. Finally, the water was removed by addition of toluene, followed by evaporation, to give a residue (190 mg) which showed partial formation of an internal ester on examination by t.l.c. in 4:5:1 butyl alcohol–acetone–water.

The internal ester was hydrolyzed by treatment of the residue, dissolved in methanol (5.5 ml), with 2M sodium hydroxide (0.5 ml). The mixture was kept for 3 h at room temperature, and then diluted with water (6 ml). Amberlite IRC-50 (H^+) ion-exchange resin was added until a pH of approximately 6 was reached, the resin was filtered off and washed with 50% aqueous methanol, and the filtrate was evaporated to a colorless solid (138 mg, 71%) which crystallized from methanol–ethyl acetate as plates, m.p. 176-178°, $[\alpha]_D^{20}$ +149° (*c* 1.42, methanol).

Anal. Calc. for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65; O, 33.38. Found: C, 56.36; H, 6.39; N, 3.76; O, 33.31.

Attempts at preparation of benzyl 2-acetamido-3-O-[D-1-(methoxycarbonyl)-ethyl]-2-deoxy-α-D-galactopyranoside (5) A. *From* 4. — Compound 4 (288 mg) was dissolved in the minimal volume of methanol, and an excess of a freshly prepared solution of diazomethane in ether was added. The yellow color persisted from the beginning of the addition. The solution was kept at room temperature, and, after the yellow color had become very faint, an excess of diazomethane solution in ether was added. After evaporation under a stream of nitrogen, the residue (277 mg) was shown by t.l.c. in 4:5:1 butyl alcohol–acetone–water to consist mostly of unreacted starting material 4. It was dissolved in ethyl acetate containing the minimal volume of methanol, and chromatographed on silica gel (20 g). Acetone–methanol (2:1) eluted 208 mg (72%) of 4.

B. *From* 3. — A solution of compound 3 (100 mg) in 60% acetic acid (2 ml) was heated for 45 min on a boiling water-bath. After being cooled to room temperature, the solution was evaporated to a syrup, and the last traces of acetic acid and benzaldehyde were removed by addition of water followed by distillation. T.l.c. of the residue (19:1 chloroform–methanol) indicated that partial hydrolysis of the methyl ester had occurred. The residue was chromatographed on a silica-gel column (8 g). Ethyl acetate eluted 14 mg (18%) of benzyl 2-acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-galactopyranoside 4-(internal ester) (6), which was recrystallized from acetone to give needles, m.p. 229–230°, $[\alpha]_D^{20}$ +117° (*c* 0.09, chloroform).

Anal. Calc. for $C_{18}H_{23}NO_7$: C, 59.17; H, 6.34; N, 3.83. Found: C, 58.94; H, 6.28; N, 3.87.

Earlier fractions eluted with 1:1 ethyl acetate–acetone contained 5 (26 mg) as a syrup which, by t.l.c. (19:1 chloroform–methanol), had a mobility identical with that of 5. Attempts to crystallize this syrup were unsuccessful. N.m.r. data (Me_2SO-d_6, tetramethylsilane as the external standard): τ 8.88, 8.77 (3-proton doublet, $J_{2',3'}$ 7 Hz (lactyl side-chain); τ 8.28 (3-proton singlet, Ac); τ 6.97 (3-proton singlet, Me ester); and τ 2.8–3.0 (5 protons, Ph).

Later fractions, eluted with 1:1 ethyl acetate–acetone and then with methanol, contained 4 (40 mg, 50%).

Hydrolysis of **3** for 15 min increased the proportion of **5** to 64%, after chromatographic separation on a silica gel column as previously described. The amount of recovered internal ester **6** decreased to 8%, and of acid **4** to 28%. Hydrolysis of **3** for 7 min gave, after preparative t.l.c. in 47:3 chloroform–methanol, 17 mg (60%) of **5** and traces of starting material **3** and acid **4**.

2-Acetamido-3-O-(D-1-carboxyethyl)-2-deoxy-D-galactose (*N-acetylgalactomuramic acid*) (**7**). — A solution of compound **4** (174 mg) in 90% methanol (or ethanol) (25 ml) was hydrogenolyzed for 2 days at room temperature and normal pressure in the presence of 10% palladium-on-charcoal (100 mg). After filtration of the suspension, the filtrate was evaporated, and the residue (126 mg, 94%) was shown, by t.l.c. (4:5:1 butyl alcohol–acetone–water), to be homogeneous. It was dissolved in methanol and precipitated by addition of ethyl acetate, to give a colorless, amorphous product, m.p. about 150° (dec.), $[\alpha]_D^{20}$ +66° (*c* 0.67, 70% ethanol).

Anal. Calc. for $C_{11}H_{19}NO_8$: C, 45.04; H, 6.53; N, 4.78; O, 43.64. Found: C, 45.21; H, 6.83; N, 4.54; O, 43.29.

This material was shown to be homogeneous by descending paper chromatography on Whatman No. 1 paper. $R_{N\text{-}acetylmuramic\ acid}$ 0.67 in 6:4:3 butyl alcohol–pyridine–water, 0.80 in butyl alcohol–acetic acid–water (25:6:25, upper phase), and 0.88 in 5:2:2 butyl alcohol–acetic acid–water. The spots detected with the silver nitrate reagent[12] were faint, but they could be detected very conveniently by the procedure of Sharon and Seifter[13].

2-Amino-3-O-(D-1-carboxyethyl)-2-deoxy-D-galactose (**8**). — Compound **7** (122 mg) was hydrolyzed with 2.5M hydrochloric acid (3 ml) for 4 h on a boiling water-bath. The solution was decolorized with activated charcoal (Merck), and then evaporated to dryness. A small volume of water was several times added and evaporated off under vacuum, the residue (91 mg) was dissolved in a small volume of water, and the solution was shaken at 0° with an excess of Dowex-1 x8 (OH^-) ion-exchange resin. The resin was filtered off, washed with water, and then stirred with water. Hydrochloric acid (M) was added to this suspension until pH 5.7 (checked with a pH meter) was reached. T.l.c. on a cellulose MN-400 plate with 5:1:2 butyl alcohol–acetic acid–water showed an R_F 0.21 (R_F of muramic acid[14], 0.28). Descending paper-chromatography on Whatman No. 1 paper showed $R_{muramic\ acid}$ 0.73 in 5:1:2 butyl alcohol–acetic acid–water, and 0.58 in 4:5:1 butyl alcohol–ethanol–water. The spots were detected with ninhydrin.

Benzyl 2-acetamido-4,6-O-benzylidene-3-O-(L-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**9**). — A solution of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranoside (**1**, 1.06 g) was treated as described for the preparation of **2**. The D-2-chloropropionic acid was synthesized in the same way as for the L-analog. After acidification of the aqueous solution containing the sodium salt of the condensation product, the resulting precipitate (755 mg) was immediately filtered off, and extensively washed with water. The filtrate was extracted many times with chloroform, and the chloroform extracts were combined, washed with water, dried, and evaporated to give additional crystalline material (127 mg); total yield, 882 mg (70%). The

materials were combined, and recrystallized from methanol–ethyl acetate to give needles, m.p. 250–251.5°, $[\alpha]_D^{20}$ +166° (*c* 0.22, methanol), +223° (*c* 0.30, pyridine).

Anal. Calc. for $C_{25}H_{20}NO_8$: C, 63.68; H, 6.20; N, 2.97. Found: C, 64.02; H, 6.36; N, 2.85.

Benzyl 2-acetamido-4,6-O-benzylidene-3-O-[L-1-(methoxycarbonyl)ethyl]-2-deoxy-α-D-galactopyranoside (**10**). — Compound **9** (25 mg) was dissolved in a small volume of methanol, and esterified by addition of a slight excess of diazomethane in ether. Evaporation of the solution, and crystallization of the residue from ethyl acetate, gave 18 mg (72%), m.p. 232–236°, $[\alpha]_D^{20}$ +150° (*c* 0.32, methanol).

Anal. Calc. for $C_{26}H_{31}NO_8$: C, 64.32; H, 6.44; N, 2.88; O, 26.36. Found: C, 64.24; H, 6.55; N, 2.89; O, 26.82.

Benzyl 2-acetamido-3-O-(L-1-carboxyethyl)-2-deoxy-α-D-galactopyranoside (**11**). — A solution of compound **9** (274 mg) in 60% acetic acid (5 ml) was heated for 3 h on a boiling water-bath. After being cooled to room temperature, the solution was evaporated to a glassy residue, and the last traces of acetic acid and benzaldehyde were removed by several additions of water, followed by evaporation. Removal of the water by addition of toluene, followed by evaporation, gave a colorless residue (210 mg). T.l.c. in 4:5:1 butyl alcohol–acetone–water showed the presence of a small amount of internal ester as a contaminant. Crystallization from methanol–ethyl acetate gave needles (150 mg, 71%), having a double m.p.: 199–200° and 230–232°, $[\alpha]_D^{20}$ +143° (*c* 0.31, methanol).

Anal. Calc. for $C_{18}H_{25}NO_8$: C, 56.39; H, 6.57; N, 3.65. Found: C, 56.35; H, 6.60; N, 3.65.

Benzyl 2-acetamido-3-O-[L-1-(methoxycarbonyl)ethyl]-2-deoxy-α-D-galacto-pyranoside (**12**). — Crude compound **11** (70 mg) was dissolved in the minimal volume of methanol, and an excess of ethereal diazomethane solution was added. The solution was evaporated under a stream of nitrogen at room temperature. The colorless residue (62 mg) was crystallized from acetone–ether–pentane, to give needles having a complex m.p., sintering at 202–206°, filamentous needles appearing at 209–210°, and final m.p. at 234–237°, $[\alpha]_D^{20}$ +104° (*c* 0.27, chloroform).

Anal. Calc. for $C_{19}H_{27}NO_8$: C, 57.42; H, 6.85; N, 3.52; O, 32.21. Found: C, 57.34; H, 6.92; N, 3.48; O, 32.14.

2-Acetamido-3-O-(L-1-carboxyethyl)-2-deoxy-α-D-galactose (*N-acetylisogalacto-muramic acid*) (**13**). — Hydrogenolysis of compound **12** (66 mg), as described for the preparation of **7**, gave in quantitative yield a product that was crystallized from methanol–ethyl acetate, m.p. 110–120° (slight dec.), $[\alpha]_D^{20}$ +27 (5 min)→+9° (equil., 20 h, *c* 0.59, 70% ethanol).

Anal. Calc. for $C_{11}H_{19}NO_8$: C, 45.05; H, 6.53; N, 4.78. Found: C, 45.17; H, 6.63; N, 4.71.

The material was shown to be homogeneous by descending paper-chromatography on Whatman No. 1 paper: $R_{\text{N-acetylmuramic acid}}$ 0.64 in 6:4:3 butyl alcohol–

pyridine–water, 0.75 in butyl alcohol–acetic acid–water (25:6:25, upper phase), and 0.87 in 5:2:2 butyl alcohol–acetic acid–water.

2-Amino-3-O-(L-1-carboxyethyl)-2-deoxy-α-D-galactose hydrochloride (**14**). — Compound **13** (10 mg) was hydrolyzed with 2.5M hydrochloric acid (1 ml) for 4 h on a boiling water-bath. The solution was evaporated to dryness, the residue was dissolved in a small volume of water, and the solution was evaporated to dryness; this procedure was repeated several times. The crystalline residue was recrystallized from methanol–acetone–ether to give needles (7 mg, 71%) that did not melt below 300°, $[\alpha]_D^{20}+107.5$ (5 min) → +89° (equil., 20 h, *c* 0.34, 70% ethanol); ν_{max}^{KBr} 3485, 3350, 3065, 2940, 2380, 1925, 1725, 1610, 1525, 1405, 1360, 1230, 1170, 1035, and 780 cm^{-1}.

Anal. Calc. for $C_9H_{18}ClNO_7$: C, 37.65; H, 6.31; N, 4.88. Found: C, 37.43; H, 6.40; N, 4.80.

The material was shown to be homogeneous by descending paper chromatography on Whatman No. 1 paper; $R_{muramic\ acid}$ 0.45 in 6:4:3 butyl alcohol–pyridine–water and 0.68 in 5:1:2 butyl alcohol–acetic acid–water; the spots were detected with the ninhydrin reagent.

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2-DEOXY SUGARS

PART XV. THE FOUR ISOMERIC METHYL GLYCOSIDES OF 2-DEOXY-D-*ribo*-HEXOSE*

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ABSTRACT

Brief treatment of 2-deoxy-D-*ribo*-hexose in methanol with a small proportion of hydrogen chloride resulted in a 100-% yield of crystalline methyl 2-deoxy-α,β-D-*ribo*-hexofuranoside in an anomeric ratio of 1:1, fractional recrystallization of which resulted in partial separation of the α-D anomer. The furanoside mixture was converted into its tris-*p*-nitrobenzoate, and, thence, into crystalline 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*ribo*-hexosyl bromide. The new halide underwent methanolysis to yield mainly methyl 2-deoxy-β-D-*ribo*-hexofuranoside. Methanolysis of 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexosyl bromide, followed by deacylation, gave the hitherto-unknown methyl 2-deoxy-β-D-*ribo*-hexopyranoside. Controlled hydrolysis of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside with acid gave crystalline methyl 2-deoxy-α-D-*ribo*-hexopyranoside, hitherto reported as a syrup.

INTRODUCTION

Nucleosides that contain 2-deoxy-β-D-*ribo*-hexofuranose as the carbohydrate residue are unknown, and, because of their very close structural relationship to the natural, "2-deoxy-D-ribofuranosyl" (2-deoxy-D-*erythro*-pentofuranosyl) nucleosides, their preparation for evaluation as potential anticancer compounds is warranted. A direct (and general) method was envisaged, involving, as a key intermediate, a suitably protected and reasonably stable tri-*O*-acylglycofuranosyl halide of 2-deoxy-D-*ribo*-hexose. This paper reports the preparation of crystalline 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*ribo*-hexosyl bromide, constituting an extension, to the furanoid forms of 2-deoxyhexoses, of our original discovery[1] that *p*-nitrobenzoic esters of 2-deoxy sugars lead to stable, crystalline per-*O*-acyl-2-deoxyglycosyl halides having utility in the direct synthesis of biologically important glycosides[2].

As an outgrowth of this study, all of the four isomeric methyl glycosides of 2-deoxy-D-*ribo*-hexose have been prepared, and isolated in crystalline form.

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RESULTS AND DISCUSSION

We have recently shown[3] that the direct methyl glycosidation of 2-deoxy-D-*arabino*-hexose* results in a 30–35% yield of methyl 2-deoxy-α-D-*arabino*-hexofuranoside. Although this procedure was of value in the preparation of some pyrimidine nucleosides containing 2-deoxy-β-D-*arabino*-hexofuranose as the carbohydrate residue[3b,c], the isolation of the furanoside from the glycosidation mixture was troublesome, requiring chromatographic procedures. For 2-deoxy-D-*ribo*-hexose (**1**), an alternative procedure, developed by Pacsu and Green[4], and involving demercaptalation of a dialkyl dithioacetal of **1** in an alcohol (methanol) was investigated, with a view to obtaining furanoside(s) in yields greater than that reported for 2-deoxy-D-*arabino*-hexose. The demercaptalation reaction has added advantages, in that the reaction is irreversible and there is no anomerization or conversion into pyranosides.

Treatment of 2-deoxy-D-*ribo*-hexose[5] (**1**) with α-toluenethiol gave the dibenzyl dithioacetal (**2**), and demercaptalation of **2** in methanol, with mercuric oxide and mercuric chloride, gave *ca.* 30-% yield of a crystalline compound, the composition of which agreed with that calculated for a methyl glycoside. The new glycoside (**3**) melted in a narrow range, and was homogeneous on paper and thin-layer chromato-

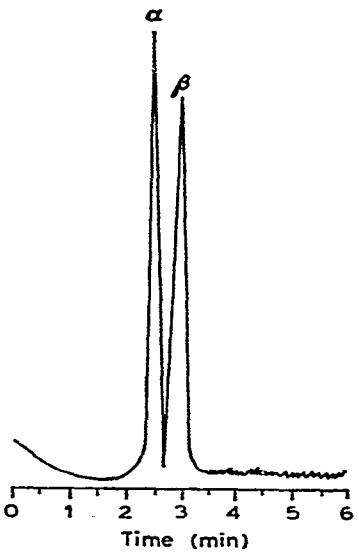


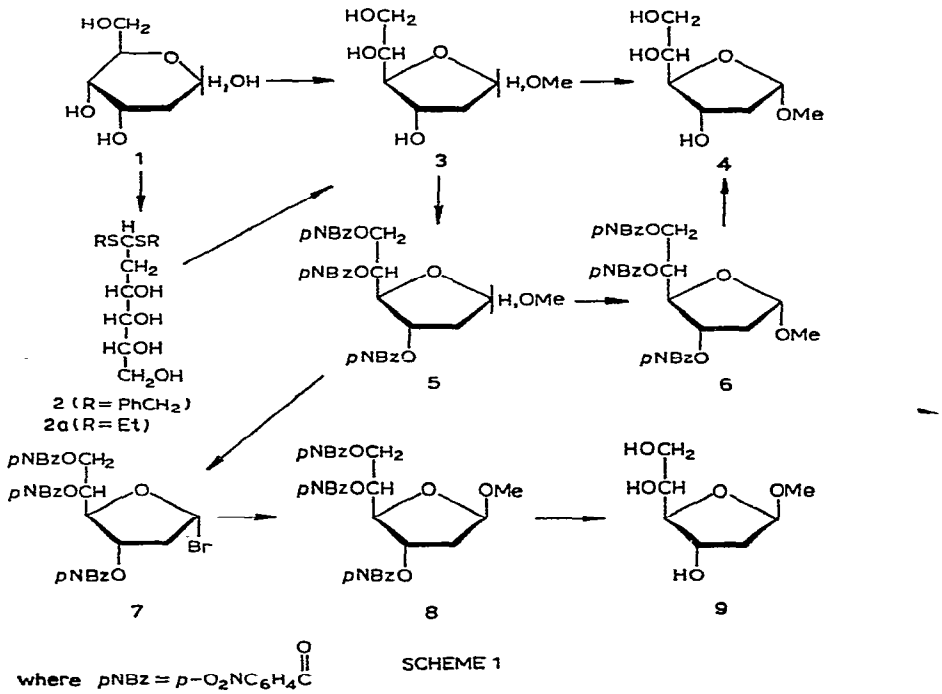
Fig. 1. G.l.c. of anomeric mixture of methyl 2-deoxy-D-*ribo*-hexofuranosides as the per(trimethylsilyl)-ated derivative.

grams, irrespective of the solvent system employed. A 60-MHz n.m.r. spectrum showed a septuplet centered at τ 5.01 for the anomeric proton(s), suggestive of a 1:1

*Similar treatment of 2-deoxy-D-*lyxo*-hexose gives only small proportions (5–10%) of furanosides, regardless of the time interval (S. J. Sexton, K. V. Bhat, and W. W. Zorbach, unpublished results).

molecular compound of the anomers of methyl 2-deoxy-D-*ribo*-hexofuranoside (**3**). The components of **3** were, nevertheless, readily separable (as their trimethylsilylated derivatives) on g.l.c. (see Fig. 1), giving peaks of approximately equal area. Both anomers were subsequently secured in pure form, and chromatographed separately, disclosing that the α-D and β-D forms have retention times of 2.5 and 3.0 min, respectively.

In order to improve the yield of furanoside by the foregoing procedure, the diethyl dithioacetal (**2a**) of **1** was prepared; when this was demercaptalated in methanol, crystalline furanoside (**3**) was readily obtained, but no improvement in the yield was noted. Considering the ease with which **3** separated, it occurred to us that the methyl glycosidation of the unsubstituted hexose (**1**) would be simpler to execute, despite the possibility that relatively low yields of furanoside (**3**) might be obtained.



SCHEME 1

2-Deoxy-D-*ribo*-hexose (**1**) was added to rapidly stirred methanol containing 0.2% of hydrogen chloride, and the reaction was followed polarimetrically. When the rotation reached a minimum (30 min), the reaction was stopped by the addition of an excess of silver carbonate. On processing the reaction mixture, a 55-% yield of product was obtained, which was identical in all respects with the furanoside (**3**) obtained through the demercaptalation procedure. Another, similar study was made, in which the reaction was stopped at different time-intervals; the results are given in Table I. The most striking feature is the yield obtained after 6 min, and, when the reaction was stopped at this point by the addition of an excess of silver carbonate,

a syrup was obtained that crystallized spontaneously to give pure methyl 2-deoxy-α,β-D-*ribo*-hexofuranoside[6] (3). At other time-intervals, the syrups obtained were (to a greater or lesser degree) impure, and it was necessary to recrystallize the products to obtain 3 relatively free from impurities.

TABLE I

METHYL GLYCOSIDATION OF 2-DEOXY-D-*ribo*-HEXOSE IN 0.2% METHANOLIC HYDROGEN CHLORIDE. OPTICAL ROTATION AND YIELD[a] OF FURANOSIDE 3 AS A FUNCTION OF TIME

Time, in min	$[\alpha]_D$, *degrees*	*Yield, %*
0	+58	0
3	+51	65
5	+45	70
6	+44	100
30	+36[b]	55
24 h	+61	15

[a]Based on recovery of crystalline material. [b]Minimum rotation.

With regard to the results shown in Table I, it is significant to note that maximum formation of furanoside does *not* occur at the point of minimum rotation, and it may be that 2-deoxy-D-*ribo*-hexose (1) is a special case, in that the furanoside (3) formed after elapse of any time-interval is, most likely, always a 1:1 anomeric mixture. Accordingly, any marked rotatory contribution provided by one anomer is offset by the other, in which event the rotatory contributions of any pyranosides present become manifest. Thus, in the present instance, a balance has been effected, as evidenced by the relatively slight change in rotation (in either direction) over a 24-h period. Also striking is the fact that the furanoside 3 is isolable (albeit in lower yield) after elapse of 24 h, a time-period after which, for other sugars, it is generally considered that most, if not all, of the furanosides has been converted into pyranosides. During the early stages of the reaction, a single spot, only*, could be observed on thin-layer chromatograms; however, after 24 h, another, slower-moving spot (*ca.* 20%) appeared, and this was subsequently identified (by comparison of R_F values) as the α-D-pyranoside (14).

Methyl 2-deoxy-D-*ribo*-hexofuranoside (3), having an ethylene glycol side-chain, should consume one mole of periodate per mole, and yield one mole of formaldehyde. Accordingly, 3 was oxidized with periodate (see Table II), and the liberated formaldehyde was estimated with chromotropic acid, giving values approaching 100% (the same as that for methyl 2-deoxy-α-D-*arabino*-hexofuranoside[3], used as a control). Similar treatment of the anomeric pyranosides (12 and 14) gave values close to zero, as expected. A second oxidation of 3 was performed, and, without isolation of the

*It was later discovered that the β-D-pyranoside co-chromatographs with 3 on paper or thin-layer sheets, irrespective of the solvent system used.

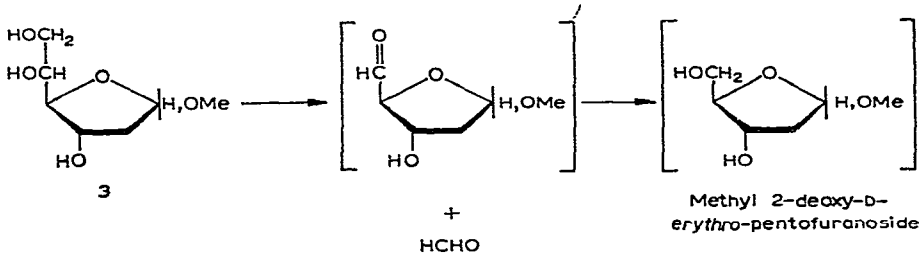
TABLE II

PERIODATE OXIDATION OF METHYL GLYCOSIDES OF 2-DEOXY-D-*ribo*-HEXOSE

Methyl glycoside of	*Conc., in μmole/ml*	*Conc. formaldehyde, in μmole/ml*	*Formaldehyde produced (%)*
2-Deoxy-α,β-D-*ribo*-hexofuranose (3)	0.25	0.22	88
2-Deoxy-α-D-*arabino*-hexofuranose[3] (control)	0.28	0.24	86
2-Deoxy-α-D-*ribo*-hexopyranose (14)	0.29	0.06[a]	0
β anomer (12)	0.23	0.04[a]	0
α-D-Glucopyranose (control)	0.52	0.03[a]	0

[a]The reading is spurious and has been disregarded.

carbohydrate product, the reaction mixture was treated with sodium borohydride. Chromatography of the latter reaction mixture revealed a single component, having an R_F value the same as that for authentic methyl 2-deoxy-D-*erythro*-pentofuranoside[8].



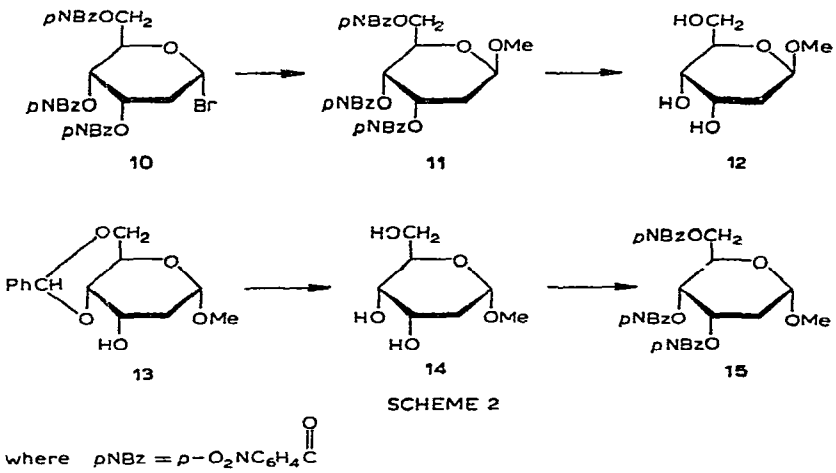
Although it appeared unlikely that the furanoside mixture (3) (behaving as a 1:1 molecular compound) could be resolved by fractional recrystallization, a partial (5%) resolution was obtained from different solvents employed in sequence. This procedure led to the isolation of pure methyl 2-deoxy-α-D-*ribo*-hexofuranoside (4). When 3 was *p*-nitrobenzoylated, the tris-*p*-nitrobenzoate (5) was obtained as an anomeric mixture, partial fractional recrystallization of which was accomplished (more readily, however, than with 3), resulting in an anomerically pure furanoside (6) having the α-D configuration, as shown by its conversion into 4 when the nitrobenzoyl groups were removed by saponification. The *p*-nitrobenzoylated furanoside (5) reacted readily with hydrogen bromide in dichloromethane to yield crystalline 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-D-*ribo*-hexosyl bromide* (7), and it is of interest that methyl 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexoside[3a,b] (C-3 epimer of the α-D anomer of 5), when treated under essentially the same conditions as given for 5, was completely unreactive. With the latter, because of the orientation of the *p*-nitrobenzoyloxy group on C-3 (on the same side of the ring as the side chain),

*The conversion of compound 3 into halide 7, *via* 5, was the subject of a preliminary communication [C. C. Bhat, K. V. Bhat, and W. W. Zorbach, *Carbohyd. Res.*, 8 (1968) 368].

there is, probably, extensive shielding about the anomeric center, whereas, with **5**, this effect would be minimized considerably.

The new halide (**7**) is very reactive, and attempts to recrystallize it resulted in decomposition. In subsequent glycosidation studies, crystalline **7** was dissolved immediately in dry dichloromethane, and the solution was added rapidly to methanol containing suspended silver carbonate, to give mainly methyl 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-β-D-*ribo*-hexoside (**8**). The crude glycoside **8** was deacylated in methanol with methoxide ion, affording a crystalline product which, by g.l.c., was shown to consist of 85% of the β-D-furanoside (**9**) and 15% of the α-D-furanoside (**4**); the β-D anomer (**9**) was readily secured in pure form by fractional recrystallization. Because of its large, positive specific rotation (+120°) and because it undergoes solvolysis (under conditions that generally lead to inverted products) to give mainly the β-D-furanoside (**8**), the halide (**7**) probably has the α-D configuration. This conclusion is, however, difficult to accept, as the bromine atom on C-1 and the *p*-nitrobenzoyloxy group on C-3 would then be on the same side of the ring, resulting in considerable conformational instability. If, indeed, **7** does have the α-D configuration, this situation could account, not only for the extreme reactivity of **7**, but also for its rapid anomerization in solution, as suggested by the formation of some α-D-furanoside during the methanolysis of **7**.

To demonstrate further that **3** does, in fact, possess the furanoid structure, unequivocal syntheses of both anomers of methyl 2-deoxy-D-*ribo*-hexopyranoside were executed (see Scheme 2). 2-Deoxy-D-*ribo*-hexose (**1**) was converted into the



SCHEME 2

where pNBz = p-$O_2NC_6H_4C(=O)$

known 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexosyl bromide[5,9] (**10**), which underwent replacement, by inversion at the anomeric center, to give methyl 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-β-D-*ribo*-hexoside (**11**). Deacylation of the protected glycoside (**11**) yielded crystalline methyl 2-deoxy-β-D-*ribo*-hexopyranoside (**12**). In a separate experiment, methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside[5] (**13**) was heated briefly with dilute acetic acid, to afford a syrup which, by t.l.c.,

was shown to consist of approximately 90% of the desired methyl 2-deoxy-α-D-*ribo*-hexopyranoside (**14**) and 10% of 2-deoxy-D-*ribo*-hexose (**1**). A resolution of the mixture was obtained by column chromatography, and, thus purified, the syrupy **14** crystallized on storage in a desiccator; it had $[\alpha]_D$ +183°. The α-D-pyranoside (**14**) was originally reported[10] as a syrup, having $[\alpha]_D$ +143°, a value that suggests that their preparation, like ours, was contamined with 2-deoxy-D-*ribo*-hexose ($[\alpha]_D$ +58°). Treatment of **14** with *p*-nitrobenzoyl chloride in pyridine gave the hitherto-unreported tris-*p*-nitrobenzoate (**15**).

With all of the four isomeric methyl glycosides of 2-deoxy-D-*ribo*-hexose now available in pure, crystalline form, it was of interest to record their n.m.r. spectra and examine the regions of the anomeric protons (see Fig. 2). As expected, the anomeric proton of the α-D-pyranoside (**14**) appeared as a triplet, centered at τ 5.31 (J 3 Hz) and, for the β-D anomer (**12**), appeared as a quadruplet at τ 5.38 ($J_{1,2}$ 9 Hz; $J_{1,2'}$ 3 Hz). The signals for the anomeric protons of both furanosides (**4** and **9**) appeared at lower fields than those of the pyranosides; that for the α-D-furanoside (**4**) is a quadruplet, centered at τ 5.05 ($J_{1,2}$ 2 Hz; $J_{1,2'}$ 3.5 Hz), and that for the β-D anomer (**9**), a triplet at τ 4.97 (J 4 Hz). The septuplet centered at τ 5.01, for the furanoside mixture (**3**), contains the signals for the α- and β-D-furanosides, and each is clearly distinguishable.

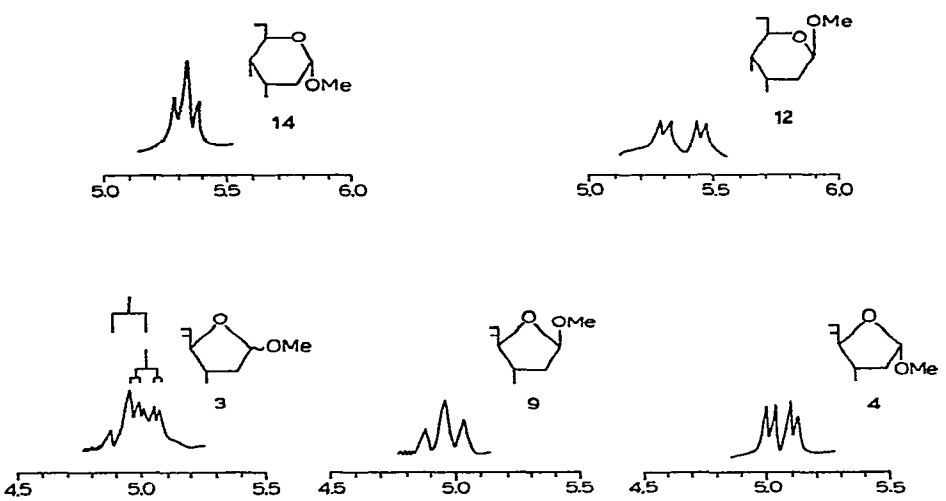
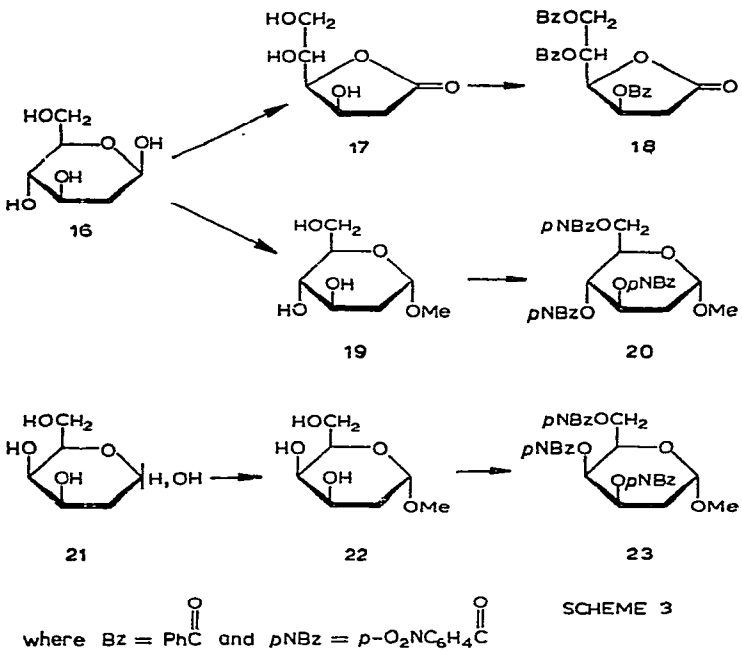


Fig. 2. N.m.r. spectra (anomeric protons) at 60 MHz of the isomeric methyl glycosides of 2-deoxy-D-*ribo*-hexose (in τ values).

Final confirmation of the furanoid structure of **3** was furnished by an unequivocal synthesis, developed by Kohn *et al.*[11], involving, as the key step, the reduction of 3,5,6-tri-*O*-benzoyl-2-deoxy-D-*ribo*-hexono-1,4-lactone[12] with disiamylborane [bis-(3-methyl-2-butyl)borane]. Much difficulty was encountered in this reduction, in which, as disclosed by t.l.c., an array of products was formed. In order to conserve the costly lactone, pilot studies were performed on 3,5,6-tri-*O*-benzoyl-2-deoxy-D-

arabino-hexono-1,4-lactone (**18**), prepared by converting 2-deoxy-D-*arabino*-hexose (**16**) into the corresponding 1,4-lactone[13] (**17**) followed by benzoylation (see Scheme 3), which afforded the lactone (**18**); this required storage in a refrigerator for three months in order to obtain it in crystalline form.

The reduction of **18** was likewise unsuccessful, and it was subsequently learned that the commercial disiamylborane used was of inferior quality*. Accordingly, the reagent was freshly prepared and, without consideration of further exploratory studies with **18**, the following synthesis was performed, in which none of the intermediates were isolated or characterized: 3,5,6-tri-*O*-benzoyl-2-deoxy-D-*ribo*-hexono-1,4-lactone[12] → 3,5,6-tri-*O*-benzoyl-2-deoxy-D-*ribo*-hexose → 3,5,6-tri-*O*-benzoyl-2-deoxy-1-*O*-*p*-nitrobenzoyl-D-*ribo*-hexose → 3,5,6-tri-*O*-benzoyl-2-deoxy-D-*ribo*-hexosyl bromide** → methyl 3,5,6-tri-*O*-benzoyl-2-deoxy-D-*ribo*-hexoside → methyl 2-deoxy-D-*ribo*-hexofuranoside. T.l.c. of the syrupy product revealed a major component (furanoside) and a minor component, identified as 2-deoxy-D-*ribo*-hexose (**1**). G.l.c. of the per(trimethylsilyl)ated mixture gave a major peak having a retention time of 3.0 min (see Fig. 1); the major component of the mixture is, therefore, methyl 2-deoxy-β-D-*ribo*-hexofuranoside (**9**).



SCHEME 3

where Bz = PhC(=O) and pNBz = p-O2NC6H4C(=O)

As a result of the work thus far described, anomeric pairs are now available for three of the four possible methyl 2-deoxyaldohexopyranosides. Save for two isomers, the corresponding *p*-nitrobenzoic esters are also available; the missing

*Personal communication from Dr. Paul Kohn.

**This conversion, and its application to nucleoside synthesis, is the subject of a forthcoming paper by P. Kohn and co-workers.

nitrobenzoylated pyranosides (**20** and **23**) were, therefore, prepared as shown in Scheme 3. Treatment of a methanol solution of 2-deoxy-D-*arabino*-hexose (**16**) with hydrogen chloride resulted in a mixture of the anomers of methyl 2-deoxy-D-*arabino*-hexopyranoside[14], from which, by fractional recrystallization, the α-D anomer (**19**) was secured in pure form*. *p*-Nitrobenzoylation of **19** resulted in the desired tris-*p*-nitrobenzoate (**20**). The methyl glycosidation of 2-deoxy-D-*lyxo*-hexose (**21**) resulted in a 70-% yield of crystalline methyl 2-deoxy-α-D-*lyxo*-hexopyranoside (**22**), considerably in excess of that originally reported[15] for this reaction, and *p*-nitrobenzoylation of **22** gave the crystalline, high-melting *p*-nitrobenzoic ester **23**.

In Table III are shown the molecular rotations of some methyl 2-deoxy-D-hexosides (furanoid and pyranoid) and their *p*-nitrobenzoates, where available. Molecular rotational differences (2A values) for anomeric pairs have been calculated; and, for the four pairs of unsubstituted glycosides shown in Table III, the values

TABLE III

MOLECULAR ROTATIONS OF SOME METHYL 2-DEOXY-D-ALDOHEXOSIDES AND THEIR *p*-NITROBENZOATES

Methyl glycoside of	[*M*]	Δ[*M*]
2-Deoxy-α-D-*arabino*-hexopyranose[14] (**19**)	+ 24,410	31,929
β anomer[14,17]	− 7,519	
2-Deoxy-α-D-*lyxo*-hexopyranose[a] (**22**)	+ 29,192	37,522
β anomer[12]	− 8,330	
2-Deoxy-α-D-*ribo*-hexopyranose[a] (**14**)	+ 32,611	36,175
β anomer[a] (**12**)	− 3,564	
2-Deoxy-α-D-*arabino*-hexofuranose[3]	+ 20,867	
2-Deoxy-α-D-*ribo*-hexofuranose[a] (**4**)	+ 26,017	34,214
β anomer[a] (**9**)	− 8,197	
2-Deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexose[a] (**20**)	− 1,312	21,018
β anomer[17]	− 22,330	
2-Deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*lyxo*-hexose[a] (**23**)	+ 10,360	32,985
β anomer[12]	− 22,625	
2-Deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexose[a] (**15**)	+141,250	44,375
β anomer[a] (**11**)	+ 96,875	
2-Deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-α-D-*arabino*-hexose[3a,b]	− 76,438	156,438
2-Deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexose[a] (**6**)	+ 80,000	

[a]This paper.

given fall well within the range for other aldose pairs[16]. For the corresponding nitrobenzoylated derivatives, however, the differences for the three anomeric pairs shown are more widely divergent, suggesting that the bulky, electronegative *p*-nitrobenzoyloxy groups increase the dissymmetry about the carbon atoms to which they are attached, resulting in anomalous Δ[M] values. The last two compounds listed in Table III constitute a pair of furanoid, C-3 epimers, having the same configuration at the anomeric center. The molecular rotational difference for these two is exceedingly

*The authors thank Dr. Gerhard Pietsch for this preparation.

large, and is particularly striking when compared with $\Delta[M] = 5150$ for the corresponding, unsubstituted furanosides. Accordingly, the two *p*-nitrobenzoylated furanosides must have highly strained structures, with much distortion of the already strained, furanoid rings, and it would be of considerable moment to have X-ray crystallographic analyses made of the two compounds.

EXPERIMENTAL

All melting points were determined with a Kofler hot-stage, optical rotations were measured with a Rudolph Model 80 polarimeter, i.r. spectra were recorded with a Perkin–Elmer Model 457 spectrophotometer, nuclear magnetic resonance (n.m.r.) spectra were recorded with a Varian Model A-60 spectrometer, g.l.c. was performed on a Micro-Tek Model 220 instrument having a dual flame detector (column packing: 2% SE-52 on Chromosorb W), and optical absorbances at 570 nm were measured with a Coleman Junior Model 6C spectrophotometer (for the estimation of formaldehyde in the periodate-oxidation studies).

Samples for t.l.c. were spotted on plates of Kiesel-Gel DF-5 (ultraviolet indicating), and developed with one of the following solvents: *A* (upper phase of 10:6:5:3 ethyl acetate–methanol–water–heptane), *B* (upper phase of 10:6:5:3 ethyl acetate–ethyl-alcohol–water–2,2,4-trimethylpentane), and *C* (upper phase of 9:6:3:1 butyl alcohol–acetic acid–ether–water). Alkaline potassium permanganate spray was used to visibilize the spots.

Samples for paper chromatography were spotted on strips of Whatman No. 1 paper, and developed with one of the following solvents: *D* (upper phase of water-saturated 9:1 butyl alcohol–toluene) and *E* (upper phase of 10:6:1:4 ethyl acetate–isobutyl alcohol–toluene–water). Carbohydrate derivatives were detected by spraying with 1% of boric acid in a solution of 1% of hydrogen chloride in 90% aqueous methanol[18].

2-Deoxy-D-ribo-*hexose dibenzyl dithioacetal* (**2**). — To 184 mg (1.12 mmoles) of 2-deoxy-D-*ribo*-hexose[5] (**1**) at 0° were added, with stirring, 0.5 ml (4 mmoles) of α-toluenethiol and 0.4 ml of concentrated hydrochloric acid. The mixture was stirred for 10 min at 0°, some crushed ice and ice–water were added, and stirring was continued for 5 min. The thick suspension was filtered, and the residue was thoroughly washed with water, and then with pentane to remove the excess of α-toluenethiol. The crystalline product was dissolved in tetrahydrofuran, the solution was dried with sodium sulfate, and the suspension filtered. The filtrate was evaporated to dryness under diminished pressure at 35°, and the resulting syrup was dissolved in tetrahydrofuran. Addition of ether resulted in the separation of 280 mg (70%) of **2**, m.p. 146.5–149°, $[\alpha]_D^{25}$ +55.5° (*c* 0.72, tetrahydrofuran).

Anal. Calc. for $C_{20}H_{26}O_4S_2$: C, 60.87; H, 6.64; S, 16.25. Found: C, 60.78; H, 6.64; S, 16.42.

2-Deoxy-D-ribo-*hexose diethyl dithioacetal* (**2a**). — To 200 mg (1.22 mmoles) of **1** at 0° were added, with stirring, 3 ml of ethanethiol and 0.4 ml of concentrated hydrochloric acid. The mixture was stirred for 30 min at 0°, and then poured, with

efficient stirring, into 100 ml of ice–water containing 1 ml of 3M sodium hydroxide (to neutralize the acid). The resulting solution was evaporated to dryness under diminished pressure at 45°, the residue was thoroughly extracted with tetrahydrofuran, and the extracts were combined, treated with Darco G-60 decolorizing carbon, and filtered, and the filtrate evaporated to a syrup. The product was crystallized from tetrahydrofuran–ether, to give 100 mg (30%) of **2a**, m.p. 93–96°, $[\alpha]_D^{25}$ +0.11° (*c* 0.92, tetrahydrofuran).

Anal. Calc. for $C_{10}H_{22}O_4S_2$: C, 44.41; H, 8.20; S, 23.72. Found: C, 44.67; H, 8.36; S, 23.87.

*Methyl 2-deoxy-α,β-*D-ribo-*hexofuranoside* (**3**). — (a) *From the dithioacetal* (**2** *or* **2a**). — To a stirred solution of 500 μmoles of either **2** or **2a** in 3 ml of anhydrous methanol were added 280 mg of mercuric chloride, 143 mg of freshly prepared, yellow mercuric oxide, and 50 mg of finely divided Drierite. The mixture was stirred for 1 h at room temperature, and filtered with the aid of a little Celite 545. To the filtrate was added 0.2 ml of pyridine, the mixture was kept in a refrigerator overnight, and the pyridine–mercuric chloride complex was removed by filtration. The filtrate was further purified by stirring it with Rexyn 300 (H^+, OH^-) ion-exchange resin* and filtering the suspension; the clear filtrate was evaporated *in vacuo* at 40° to a syrup, which partially crystallized on storage overnight in a desiccator containing phosphorus pentaoxide. Recrystallization from tetrahydrofuran–ether gave furanoside **3**, m.p. 93–95°, $[\alpha]_D^{25}$ +39.2° (*c* 0.473, methanol). Yields of crystalline **3** varied from 30–34%.

Anal. Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.92. Found: C, 46.98; H, 7.79.

(b) *From the methyl glycosidation of 2-deoxy-*D-ribo-*hexose*[6] (**1**). — To 60 ml of a 0.2% solution of hydrogen chloride in methanol was added 1.0 g (6.1 mmoles) of finely powdered 2-deoxy-D-*ribo*-hexose (**1**), and the reaction mixture was stirred at room temperature for 6 min. An excess (6 g) of silver carbonate was quickly added, and stirring was continued for 15 min, after which, the suspension was filtered through a bed of decolorizing carbon. Evaporation of the filtrate gave a syrup that crystallized rapidly, the product (1.08 g) having m.p. 93–95°. It was homogeneous on paper chromatograms with solvent *D* (R_F 0.47) and with solvent *E* (R_F 0.27); in each case, the R_F value was identical with that of the methyl 2-deoxy-α,β-D-*ribo*-hexofuranoside (**3**) obtained in the preceding preparation. Recrystallization from tetrahydrofuran–pentane gave 910 mg (85%), with no change in m.p.; the total yield of pure **3** was, therefore, 100%.

*Methyl 2-deoxy-α-*D-ribo-*hexofuranoside* (**4**). — Methyl 2-deoxy-α,β-D-*ribo*-hexofuranoside (1.9 g), prepared as described in the preceding experiment, was recrystallized once from tetrahydrofuran–ether, twice from ethyl alcohol–ether–pentane, and three times from ethyl acetate, to give 100 mg (5%) of **4**, m.p. 115–119°, $[\alpha]_D^{25}$ +146° (*c* 0.582, methanol). The trimethylsilylated derivative of **4** appeared, on g.l.c., as a single peak having a retention time of 2.5 min (see Fig. 1).

*Fisher Scientific Co.

*Methyl 2-deoxy-3,5,6-tri-O-*p*-nitrobenzoyl-α,β-*D*-ribo-hexoside* (**5**) *and the pure α-*D* anomer* (**6**). — To a stirred suspension of 425 mg (2.4 mmoles) of *p*-nitrobenzoyl chloride in 6 ml of anhydrous pyridine at 0° was added 100 mg (560 μmoles) of the furanoside (**3**). Stirring was continued for 1 h at 0° and for 1 h at room temperature. The mixture was kept in a refrigerator for 3 days, and then stirred for 1 h at room temperature; 10 ml of saturated, aqueous sodium hydrogen carbonate was now carefully added. The resulting mixture was poured, with stirring, into 200 ml of ice–water, stirring was continued for 1.5 h, and the product that separated was filtered off by suction and washed several times with water. After being dried in a vacuum desiccator containing phosphorus pentaoxide, the solid was crystallized from acetone–ether–pentane to give 200 mg (57%) of **5**, m.p. 137–147°.

Fractional recrystallization of the anomeric mixture (**5**) from tetrahydrofuran–ether–pentane yielded 70 mg (20%) of pure methyl 2-deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexoside (**6**), m.p. 152–154°, $[\alpha]_D^{25}$ +128° (*c* 0.456, acetone).

Anal. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found: C, 53.96; H, 3.81; N, 6.69.

Conversion of **6** *into the α-*D*-furanoside* (**4**). — A suspension of 50 mg (80 μmoles) of **6** in 5 ml of 20 mM methanolic sodium methoxide was stirred for 24 h at room temperature. The solvent was evaporated off at 40° under diminished pressure, and the residue was suspended in 5 ml of water. The suspension was washed with three 5-ml portions of ether, the aqueous layer was stirred for 7 min with a small portion of Rexyn 300 (H^+, OH^-) ion-exchange resin, the suspension was filtered, and the clear filtrate was evaporated to dryness under diminished pressure at 50°. The resulting syrup was dried by co-evaporation with absolute ethyl alcohol; it was then dissolved in a small volume of absolute ethyl alcohol, and ether–pentane was added to incipient turbidity; yield of **4**, 10 mg (70%), m.p. 115–119°. It was homogeneous by g.l.c. of its tris(trimethylsilyl) ether, which had a retention time of 2.5 min (see Fig. 1).

*2-Deoxy-3,5,6-tri-*O*-*p*-nitrobenzoyl-α-*D*-ribo-hexosyl bromide* (**7**). — To 105 mg (170 μmoles) of the tris-*p*-nitrobenzoate (**5**) was added 3.5 ml of a saturated solution of hydrogen bromide in dichloromethane. The solution was kept for 12 min at room temperature under rigorous exclusion of moisture; it was concentrated to half its volume by evaporation under diminished pressure (25°), and diluted with ether, and pentane was added to incipient turbidity. The solution was refrigerated for 2 h, and the resulting crystals were filtered off, and washed with dry ether, to give 85 mg (79%) of the bromide **7**, m.p. 93–95° (dec. 135°), $[\alpha]_D^{25}$ +120° (*c* 0.489, dichloromethane). The halide gave a strong, positive Beilstein test.

*Methyl 2-deoxy-β-*D*-ribo-hexofuranoside* (**9**). — A solution of 500 mg (740 μmoles) of the halide **7** in 15 ml of dry dichloromethane was added to a stirred suspension of 2 g of silver carbonate in 70 ml of anhydrous methanol. The mixture was stirred for 2 h, and filtered, and the silver salts were washed with hot dichloromethane. The filtrate and washing were combined and evaporated under diminished pressure to give 270 mg of the *p*-nitrobenzoylated furanoside (**8**) as syrupy material, which could not be brought to a satisfactory crystalline state. Without further processing,

the crude **8** was treated with 20 ml of 20 mM methanolic sodium methoxide, the mixture was stirred for 24 h at room temperature, and the solvent was evaporated off under diminished pressure at 40°. The residue was suspended in 15 ml of water, the suspension was washed with three 10-ml portions of ether, and the aqueous layer was stirred for 7 min with a small portion of Rexyn 300 (H^+, OH^-) ion-exchange resin. The resin was filtered off, and the filtrate was evaporated to dryness at 45° to afford a syrup that crystallized spontaneously; yield 50 mg (37% based on **7**). The product, as shown by g.l.c. of its per(trimethylsilyl) ether, consisted of 85% of the β-D anomer (**9**) and 15% of the α-D anomer (**4**). Fractional recrystallization, first from tetrahydrofuran–ether–pentane, and then from ethyl acetate, gave pure β-D-furanoside (**9**), m.p. 117–122°, $[\alpha]_D^{25}$ −46° (*c* 0.20, methanol). The product, thus purified, gave a single peak on g.l.c. of its per(trimethylsilyl) ether, which had a retention time of 3.0 min (see Fig. 1); consequently, an elementary analysis was not performed.

Methyl 2-deoxy-3,4,6-tri-O-p-*nitrobenzoyl-β*-D-ribo-*hexoside* (**11**). — A solution of 1.87 g (2.78 mmoles) of 2-deoxy-3,4,6-tri-*O*-*p*-nitrobenzoyl-α-D-*ribo*-hexosyl bromide[5,9] (**10**) in 20 ml of dry dichloromethane was added to a stirred suspension of 2 g of silver carbonate in 100 ml of absolute methanol. The mixture was stirred for 2 h, and filtered, and the silver salts were washed with hot dichloromethane. The filtrate and washings were combined, and evaporated to dryness under diminished pressure, and the residue was twice recrystallized from acetone–pentane to give 700 mg (40%) of pure **11**, m.p. 209–211°, $[\alpha]_D^{25}$ +155° (*c* 0.619, acetone).

Anal. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72 H, 3.71; N, 6.72. Found: C, 53.93; H, 3.76; N, 6.53.

Methyl 2-deoxy-β-D-ribo-*hexopyranoside* (**12**). — A suspension of 645 mg (1.04 mmoles) of **11** in 25 ml of 20 mM methanolic sodium methoxide was stirred for 24 h at room temperature. The solvent was removed under diminished pressure at 40°, and the residue was suspended in 40 ml of water. The suspension was washed with three 15-ml portions of ether, and the aqueous layer was stirred for 7 min with 3 g of Rexyn 300 (H^+, OH^-) ion-exchange resin. The resin was filtered off, the filtrate was evaporated to dryness under diminished pressure at 50°, and the resulting syrup was dried once by co-evaporation with absolute ethyl alcohol, whereupon it crystallized spontaneously. The product was recrystallized from tetrahydrofuran–pentane to give 110 mg (60%) of methyl 2-deoxy-β-D-*ribo*-hexopyranoside (**12**), m.p. 99–100.5°, $[\alpha]_D^{25}$ −20° (*c* 0.52, methanol).

Anal. Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.92. Found: C, 47.30; H, 8.09.

Methyl 2-deoxy-α-D-ribo-*hexopyranoside*[10] (**14**). — A suspension of 2.6 g (10 mmoles) of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*ribo*-hexopyranoside[5] (**13**) in 150 ml of 0.1M acetic acid was stirred for 24 min at 80°. The acid was neutralized with 3M sodium hydroxide, the solution was washed with three 50-ml portions of chloroform, and the aqueous layer was stirred for 10 min with 25 g of Rexyn 300 (H^+, OH^-) ion-exchange resin. The suspension was filtered, and the filtrate was evaporated to a syrup, which was chromatographed on a column (4 × 40 cm) of

cellulose powder by elution with solvent *D*. Collection of fractions (3 ml each) was begun when the effluent gave a faint, positive reaction with a boric acid reagent[18], and the product (**14**) was recovered as a syrup from the first 136 tubes. It was homogeneous on paper chromatograms (solvent *D*), and had R_F 0.38; yield, 1.0 g (55%). On storage of the syrup for several days in a desiccator containing phosphorus pentaoxide, the product crystallized, and recrystallization from tetrahydrofuran–ether afforded pure **14** as slightly hygroscopic needles, m.p. 97–99.5°, $[\alpha]_D^{25}$ +183° (*c* 0.521, methanol).

Anal. Calc. for $C_7H_{14}O_5$: C, 47.19; H, 7.92. Found: C, 47.16; H, 8.13.

Methyl 2-deoxy-3,4,6-tri-O-p-*nitrobenzoyl*-α-D-ribo-*hexoside* (**15**). — To 259 mg (1.45 mmoles) of the α-D-pyranoside (**14**) in 10 ml of anhydrous pyridine at 0° was added, with stirring, 1.1 g (6.1 mmoles) of *p*-nitrobenzoyl chloride. Stirring was continued for 1 h at 0° and for 1 h at room temperature, and the mixture was kept in a refrigerator for 3 days. It was then stirred for 1 h at room temperature, and 15 ml of saturated, aqueous sodium hydrogen carbonate was then carefully added. The mixture was poured, with stirring, into 300 ml of ice–water, stirring was continued for 1.5 h, and the product that separated was filtered off, and washed well with water. The solid was dried in a vacuum desiccator (phosphorus pentaoxide) and then recrystallized three times from tetrahydrofuran–ether, to yield 450 mg (50%) of **15**, m.p. 136–140°, $[\alpha]_D^{25}$ +226° (*c* 0.48, acetone).

Anal. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found: C, 53.55; H, 3.56; N, 6.58.

3,5,6-Tri-O-*benzoyl-2-deoxy*-D-arabino-*hexono-1,4-lactone* (**18**). — To 488 mg (3.0 mmoles) of 2-deoxy-D-*arabino*-hexono-1,4-lactone[13] (**17**) in 4 ml of dry pyridine at 0° was added dropwise 2.2 ml (20 mmoles) of benzoyl chloride. The mixture was stirred for 1 h at room temperature, and then kept in a refrigerator for 3 days. The excess of benzoyl chloride was decomposed by careful addition of an excess of saturated, aqueous sodium hydrogen carbonate, and the mixture was poured, with stirring, into 200 ml of ice–water. The gummy solid that separated was filtered off, washed with water, and dissolved in acetone. The solution was decolorized with Darco G-60 decolorizing carbon, the suspension was filtered, and the filtrate was evaporated under diminished pressure to a clear syrup which was homogeneous on t.l.c. (R_F 0.79) with solvent *B*. The syrup was dissolved in ethyl alcohol, and the solution was kept in a refrigerator for three months, after which time, the product crystallized slowly. The crude product (1 g) was recrystallized three times from tetrahydrofuran–pentane to give 400 mg (36%) of analytically pure **18**, m.p. 73–78°, $[\alpha]_D^{25}$ −128° (*c* 0.975, acetone); ν_{max}^{KBr} 1799 (γ-lactone) and 1730 cm^{-1} (benzoate carbonyl).

Anal. Calc. for $C_{27}H_{22}O_8$: C, 68.35; H, 4.67. Found: C, 68.51; H, 4.77.

Methyl 2-deoxy-3,4,6-tri-O-p-*nitrobenzoyl*-α-D-arabino-*hexoside* (**20**). — To a stirred suspension of 3.9 g (21 mmoles) of *p*-nitrobenzoyl chloride in 30 ml of anhydrous pyridine at 0° was added 990 mg (5.6 mmoles) of methyl 2-deoxy-α-D-*arabino*-hexopyranoside[14] (**19**). Stirring was continued for 1 h at 0° and for 1 h at

room temperature, and the mixture was kept in a refrigerator for 3 days. It was then stirred for 1 h at room temperature, and 25 ml of satured, aqueous sodium hydrogen carbonate was carefully added. The resulting mixture was poured, with stirring, into 500 ml of ice–water. Stirring was continued for 1 h, and the product that separated was filtered off, and washed several times with water. The solid was dried in a vacuum desiccator (phosphorus pentaoxide), and recrystallized from acetone–ether to afford 1.4 g (40%) of **20**, containing one molecule of acetone of crystallization per molecule, m.p. 100–110°, $[\alpha]_D^{25}$ −2.1° (*c* 0.725, acetone). After being dried for 2 days at 110°/0.1 torr, the product* (**20**) melted at 150°.

Anal. Calc. $C_{28}H_{23}N_3O_{14}\cdot C_3H_6O$: C, 54.50; H, 4.25; N, 6.15. Found: C, 55.11; H, 4.19; N, 6.44. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found: C, 53.80; H, 3.61; N, 6.71.

*Methyl 2-deoxy-α-*D-lyxo-*hexopyranoside* (**22**). — A solution of 410 mg (2.5 mmoles) of 2-deoxy-D-*lyxo*-hexose** (**21**) in 10 ml of 2.5% methanolic hydrogen chloride was kept for 48 h at room temperature. An excess (1.5 g) of silver carbonate was added, the suspension was stirred for 10 min, and filtered through a bed of Darco G-60 decolorizing carbon, and the filtrate was evaporated to dryness under diminished pressure at 40°. The resulting syrup was crystallized from ethyl acetate–pentane, and two recrystallizations from the same solvent mixture gave 310 mg (70%) of pure **22**, m.p. 115–116°, $[\alpha]_D^{24}$ +164° (lit.[15] m.p. 112–113°, $[\alpha]_D$ +170°).

*Methyl 2-deoxy-3,4,6-tri-*O-p-*nitrobenzoyl-α-*D-lyxo-*hexoside* (**23**). — To a mixture of 12 ml of dry pyridine and 1.113 g (6 mmoles) of *p*-nitrobenzoyl chloride was added 267 mg (1.5 mmoles) of **22**, and the mixture was stirred for 1 h at 0°. It was kept in a refrigerator for 48 h, and then stirred for 1 h at room temperature, and the excess of the acid chloride was decomposed by stirring the mixture with 6 ml of saturated, aqueous sodium hydrogen carbonate. The resulting suspension was poured, with efficient stirring, into 200 ml of ice–water, and stirring was continued until all of the ice had melted. The precipitate was filtered off, dried in air, and then in a vacuum desiccator (phosphorus pentaoxide), and recrystallized from dichloromethane–ether, to afford 750 mg (80%) of **23**, m.p. 178–179°, $[\alpha]_D^{24}$ +58.2° (*c* 1.048, dichloromethane).

Anal. Calc. for $C_{28}H_{23}N_3O_{14}$: C, 53.72; H, 3.71; N, 6.72. Found: C, 53.50; H, 3.50; N, 6.93.

Periodate oxidation studies[7]. — To a weighed amount (50–100 μmoles) of the compound to be oxidized, in 0.5 ml of water, were added 2 ml of 0.3M periodic acid and 2 ml of M sodium hydrogen carbonate. The mixture was kept for 1 h at room temperature, and then 15 ml of 0.5M sulfuric acid and 5 ml of M sodium arsenite were added. The mixture was gently agitated until the iodine that was liberated on adding the sodium arsenite had disappeared, and it was then diluted with water,

*Attempts to recrystallize desolvated **20** from acetone–ether resulted in solvated material, m.p. 100–110°.

**Aldrich Chemical Co., Inc., Milwaukee, Wisconsin.

so that the concentration of formaldehyde was less than 600 nmoles per ml. One ml of the diluted solution was mixed with 10 ml of chromotropic acid reagent* in a test tube. The test tube was stoppered, heated for 30 min in a boiling-water bath, and cooled; the absorbance of the sample was measured against that of a blank, at 570 nm.

Standard formaldehyde solutions were prepared from samples of pure D-glucose (U. S. National Bureau of Standards) by following the procedure described above.

Methyl 2-deoxy-D-erythro-*pentofuranoside.* — To 7 mg (39 μmoles) of methyl 2-deoxy-D-*ribo*-hexofuranoside (3) in 0.5 ml of water were added 2 ml of 0.3M periodic acid and 2 ml of M sodium hydrogen carbonate. The mixture was kept for 1 h at room temperature, 20 mg of sodium borohydride was added, and the mixture was shaken gently for a few min. The excess of sodium borohydride was neutralized with 0.1M sulfuric acid, and the solution was examined by t.l.c. with solvent *A*, revealing a single component having an R_F value (0.23) identical with that of authentic methyl 2-deoxy-D-*erythro*-pentofuranoside[8].

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*The reagent was prepared by dissolving 1 g of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) in 100 ml of water and adding sufficient 12.5M sulfuric acid to bring the total volume to 500 ml.

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THE SPECIFIC SUBSTANCE FROM *Diplococcus pneumoniae* TYPE 34 (U. S. TYPE 41). SYNTHESIS OF METHYL 3-*O*-β-D-GALACTOFURANOSYL-α-D-GLUCOPYRANOSIDE

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ABSTRACT

Methyl 3-*O*-β-D-galactofuranosyl-α-D-glucopyranoside has been synthesized. In 1.25% overall concentration, it does not inhibit the homologous precipitin reaction of *D. pneumoniae* S-34 with its rabbit serum.

INTRODUCTION

The structure of the capsular polysaccharide of *Diplococcus pneumoniae* Type 34 has been elucidated in detail[1-4]. The antigen contains a phosphodiester linkage, *O*-acetyl groups, and a variety of hexose residues in both the furanose and pyranose form; it is, therefore, an excellent material for the study of the immunodominance of its various structural features.

RESULTS AND DISCUSSION

The structure[1,2] of S-34 is given in Fig. 1. Previously, the immunoimportance of the *O*-acyl group had been shown[3], and we have identified this group[4] as an *O*-acetyl group that is located on O-6 of the D-galactofuranosyl residue marked C in Fig. 1. This is not the first example of the great influence that *O*-acyl groups can have on the proportion of antibody precipitated. Heidelberger[5] has shown that, when S-18 is deacetylated, the product precipitates only about 20% of the antibodies from a homologous horse-serum.

This communication describes the preparation of methyl 3-*O*-β-D-galactofuranosyl-α-D-glucopyranoside (**1**) and compares its inhibitory power on the S-34/anti 34 system with that of the previously prepared[4] methyl 6-*O*-acetyl-β-D-galactofuranoside. The disaccharide under consideration occurs in S-34 as A–B (see Fig. 1). The methyl α-D-glycoside **1** was prepared in order to approach as closely as possible the structural features of the linkage between B and C in the native antigen. When the same rabbit anti *D. pn.* 34 as before was used, together with the same molar concentration of compound **1** as had been used[4] for methyl 6-*O*-acetyl-β-D-galacto-

furanoside, there was no (or negligible) inhibition of the S-34/rabbit anti S-34 precipitin system. The repeating units of Type 34 pneumococcal polysaccharide are linked together by a phosphodiester bridge between the primary hydroxyl group of residue E (ribitol) of one unit and the 3-hydroxyl group of residue A (D-galacto-

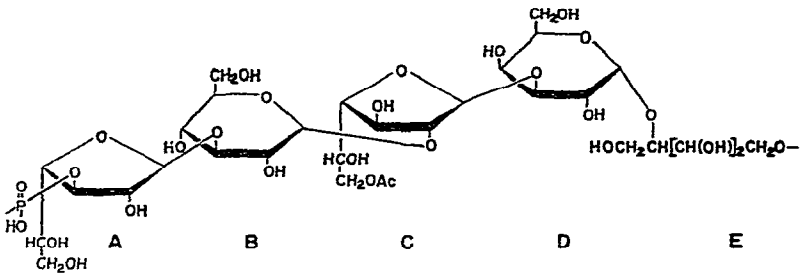


Fig. 1. Repeating unit of S-34.

furanose) of the next unit[6]. That glycoside **1** does not inhibit the homologous precipitin reaction (as does methyl 6-*O*-acetyl-*β*-D-galactofuranoside[4]) may be significant. It has been proposed[6] that phosphates of sugars could direct part of the immunochemical interaction of antibodies to[7] RNA. It will be interesting to see if the S-34 precipitin system can be inhibited more by phosphate ester derivatives of D-galactofuranose; this matter is now under investigation in this laboratory.

The conventional preparation of **1** by condensation of tetra-*O*-acetyl-*β*-D-galactofuranosyl chloride[8] (**2**) with a suitably protected derivative of D-glucopyranose leads to difficulties, as chloride **2** has a tendency to yield orthoester derivatives, instead of true disaccharides, in the Koenigs–Knorr synthesis. Consequently, a method suggested by Kochetkov and co-workers[9] was followed (see Fig. 2), employing

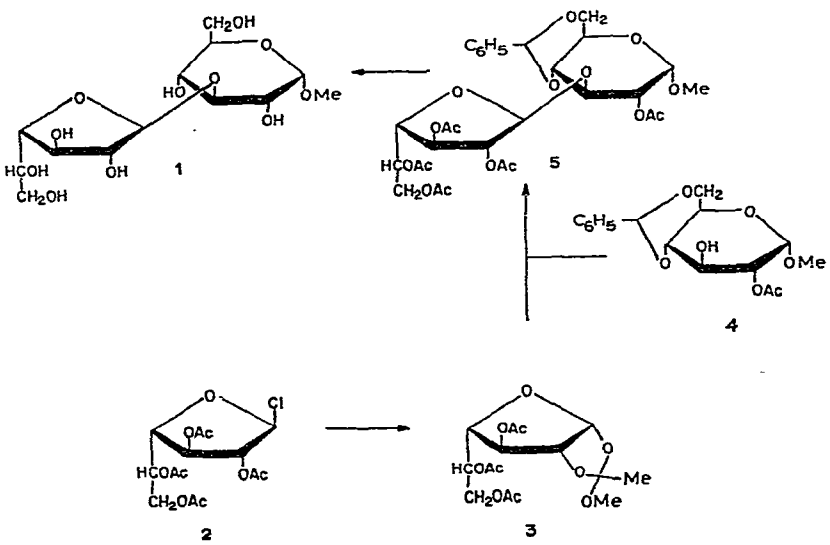


Fig. 2

3,5,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-α-D-galactofuranose[9] (**3**) for condensation with methyl 2-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside[10] (**4**).

Our preparation of **3** was found to retain lutidine tenaciously, even after chromatography on aluminum oxide[9]; its removal appeared to be imperative, as this base could inactivate the mercuric bromide catalyst. It was found that an excellent way of removing contaminating lutidine was to pass **3** rapidly through a column of silica gel (70–325 mesh; E. Merck, Darmstadt). The n.m.r. spectrum of samples of **3** thus purified showed complete purity, and indicated the presence of three acetyl groups, one *C*-methyl group, one *O*-methyl group, and one β-hydrogen atom at C-1 (centered around τ 4.0).

Condensation of **3** with **4** afforded crystalline methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-*O*-(tetra-*O*-acetyl-β-D-galactofuranosyl)-α-D-glucopyranoside (**5**), which was reductively debenzylidenated and then deacetylated, to yield, after purification by column chromatography on charcoal, crystalline **1**. Oxidation with periodate (4 moles per mole) led to production of iodine, and gave a solution containing, after hydrolysis, only a glucose. The hydrogen atom on C-4 of the D-galactofuranosyl group in **1** can be oxidized, leading to over-oxidation; and, hence, periodate oxidation is not a reliable way of confirming the structure. Consequently, **1** was methylated directly[11], and the product was methanolyzed. Upon g.l.c. of the methylated methyl glycosides, three major peaks were found: one for a methyl tetra-*O*-methylgalactofuranoside and two for the anomers of methyl 2,4,6-tri-*O*-methylglucoside. Under the conditions used, it was possible to distinguish between methyl 2,4,6- and 3,4,6-tri-*O*-methylglucoside, and the methylation experiment thus shows the absence of acetyl migration in **4** during its reaction with* **3**.

The 100-MHz n.m.r. spectrum of **5** shows a number of readily recognizable features (see Fig. 3a). First of all, it shows the presence of five *O*-acetyl groups. Also, there are 8 protons in the region τ 5.6–6.5. These have received the following assignments: 2 H-6(Gal) + H-4(Gal) + 2 H-6(G) + H-4(G) + H-5(G) + H-3(G). The signals (4 H) between τ 5.0 and 5.3 are assigned to the protons on those carbon atoms in **5** that bear an acetoxy group; these are H-5(Gal) + H-3(Gal) + H-2(Gal) + H-2(G). The multiplet at τ 4.7–4.9 represents the two anomeric protons; this appears to be a singlet [for H-1(Gal)] superimposed on a multiplet. The assignment

*Compound **3** was also condensed[9] with 1,2,4,6-tetra-*O*-acetyl-β-D-glucose (**6**). Acetyl migration in **6** may occur to a small extent during the reaction, as a fraction moving faster than **6** on t.l.c., but yielding only a glucose upon deacetylation, was isolated. Hence, this must be a tetra-*O*-acetyl-D-glucose different from **6**. The mixture was separated into its components by extensive chromatography, and there was obtained a fraction which, when deacetylated, yielded a galactose, a glucose, and a disaccharide. The disaccharide was obtained crystalline (m.p. 153–165°) following chromatography on charcoal. It had the correct elemental analysis, gave a glucose and a galactose upon hydrolysis, and the retention time on g.l.c. of its per(trimethylsilyl) ether was similar to the retention time for the per(trimethylsilyl) ether of **1**. There is good reason to believe (methylation studies) that the compound is 3-*O*-β-D-galactofuranosyl-D-glucose (**7**), but this could not be unequivocally shown. The synthesis of **7** is more profitably undertaken by a different route, involving the recently reported[12] benzyl 2-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucoside.

of a singlet at τ 4.76 to H-1(Gal) seems reasonable. The 100-MHz n.m.r. spectrum of penta-*O*-acetyl-β-D-galactofuranose (see Fig. 3b) shows H-1 as a singlet at τ 3.8, as the *O*-acetyl group at O-1 has a much more deshielding effect than has the D-glucose moiety in **5**. The 100-MHz spectrum of methyl tetra-*O*-acetyl-β-D-galactofuranoside (see Fig. 3c) shows H-1 as a singlet at τ 5.1; this, too, is acceptable, as the substituted D-glucose moiety in **5** would be expected to deshield H-1(Gal)

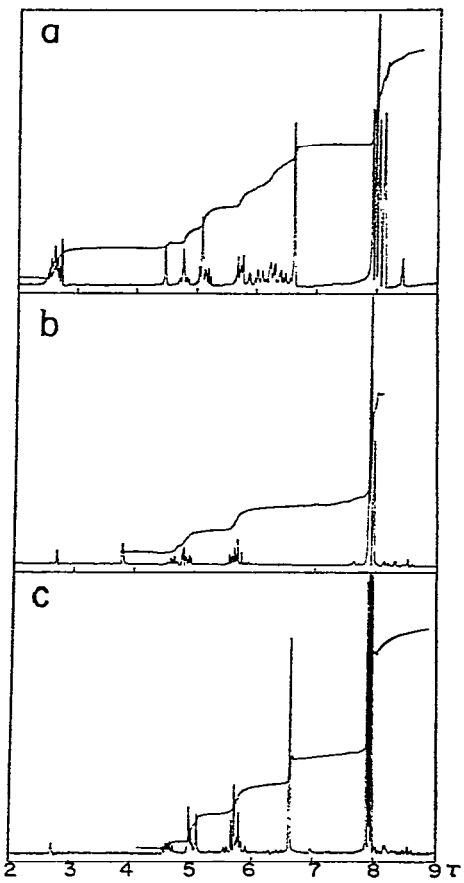


Fig. 3. 100-MHz n.m.r. spectra ($CDCl_3$) of (a) compound **5**, (b) penta-*O*-acetyl-β-D-galactofuranose, and (c) methyl tetra-*O*-acetyl-β-D-galactofuranoside.

more than would a methoxyl group. There is, however, one uncertainty. One proton, showing as the multiplet at τ 4.7–4.9 upon which the H-1(Gal) singlet is superimposed, may be H-3(Gal) instead of H-1(G). Stevens and Fletcher[13] have shown that H-3 in esterified furanose derivatives usually shows up at lower field than H-2, and, indeed, there is a multiplet for H-3 in the spectra for both methyl tetra-*O*-acetyl-β-D-galactofuranoside and penta-*O*-acetyl-β-D-galactofuranose, both at τ 4.55–4.75. If, however,

the τ 4.7–4.9 area in the spectrum of **5** is assigned to H-3(Gal) + H-1(Gal), it follows that H-1(G) must be assigned to the 4-proton multiplet at τ 5.05–5.25.

EXPERIMENTAL

Methyl 3-O-β-D-galactofuranosyl-α-D-glucopyranoside. — β-D-Galactofuranose pentaacetate (5 g, 14.5 mmoles, $[\alpha]_D^{20}$ −42.6°), was converted into the crystalline chloride [8], and this was dissolved in dichloromethane (10 ml). 2,6-Lutidine (3 ml) was added as described by Kochetkov and co-workers[9], and, after 15 min, methanol (10 ml) was added. After 24 h, dichloromethane was added, and the solution was washed with ice water (the first washing being extracted with dichloromethane), and dried (sodium sulfate). T.l.c. on Alumina G plates [3:1 (v/v) benzene–ether] indicated the presence of a small amount of almost stationary material. On silica gel [1:1 (v/v) benzene–ether], a major spot was observed that traveled slightly slower than methyl β-D-galactofuranoside tetraacetate, and a minor spot traveling at half that rate. The material was twice passed rapidly through a column of silica gel [1:2 (v/v) benzene–ether], and the major fraction was collected; it was free from any trace of lutidine. It was dried for 24 h under high vacuum, to give syrupy 3,5,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-α-D-galactofuranose (**3**) (3.172 g; 68.5%, based on the pentaacetate). The n.m.r. spectrum identified it unequivocally as being the orthoacetate.

The orthoester **3** (1.6 g, 3.8 mmoles) and methyl 2-*O*-acetyl-4,6-*O*-benzylidene-α-D-glucopyranoside[10] (1.35 g, 3.7 mmoles) were dissolved in nitromethane (17 ml, purified), and the reaction was allowed to proceed under the catalysis of mercuric bromide (90 mg) as described by Kochetkov and co-workers[9]. The mixture was cooled, and pyridine (0.25 ml) was added. Concentration under vacuum gave an oil that was resolved into its components by chromatography on silica gel with 4:1 (v/v) benzene–ether. One fraction crystallized; recrystallization yielded pure methyl 2-*O*-acetyl-4,6-*O*-benzylidene-3-*O*-(2,3,5,6-tetra-*O*-acetyl-β-D-galactofuranosyl)-α-D-glucoside (**5**), 401 mg (14%), m.p. 140–143°, $[\alpha]_D^{20}$ +37° (*c* 0.5, ethyl alcohol).

Anal. Calc. for $C_{30}H_{38}O_{16}$: C, 55.04; H, 5.85. Found: C, 54.80; H, 6.11.

The fully protected disaccharide **5** (400 mg; 530 μmoles) was reductively debenzylidenated in ethyl alcohol (80 ml) in the presence of freshly prepared palladium black. The product was deacetylated with 33 mM sodium methoxide in methanol, and, after neutralization with Dry Ice, the solution was evaporated to a syrup, which was added to the top of a column of carbon (Nuchar, prewashed successively with hydrochloric acid, water, ethyl alcohol, and water), and the disaccharide was eluted with 1:9 (v/v) ethyl alcohol–water; yield, 132 mg (70%).

The compound crystallized, and, after recrystallization from ethyl alcohol, had m.p. 148.5–150°, $[\alpha]_D^{20}$ +18.3° (*c* 0.5, water). The per(trimethylsilyl) ether showed a single peak upon g.l.c. (3% SE 52 on Gaschrom A).

Anal. Calc. for $C_{13}H_{24}O_{11}$: C, 43.82; H, 6.79. Found: C, 43.93; H, 6.84.

Methylation of compound **1**. — Compound **1** (5 mg; 14 μmoles) was dried over calcium sulfate under high vacuum, and then methylated in dry *N,N*-dimethyl-

formamide (1.25 ml) with methyl iodide (0.25 ml) and silver oxide (250 mg) in the presence of Drierite (250 mg). After three days, the mixture was processed as usual, and the resultant material was methanolyzed with 2% hydrogen chloride in methanol for 6 h under reflux. Pyridine was added, and the solution was evaporated to an oil, which was examined by g.l.c. on a column (6.5 ft) of 15% DEGS on Chromosorb W (AW). One peak for a methyl tetra-*O*-methylgalactofuranoside and 2 other peaks (cochromatographing with methanolyzed, authentic 2,4,6-tri-*O*-methyl-D-glucose) were observed.

Immunochemical behavior of the disaccharide. — Rabbit serum* against *D. pneumoniae* Type 34 (U. S. Type 41) was diluted tenfold, and set up, as before[4], with a 0.01% solution of S-34 in 0.85% saline. The serum used here had been absorbed with C-substance from *D. pn.* Type IV (antibody to C-substance was not observed to be present). The precipitated antibody–antigen complex was washed, and analyzed as before[4]. The results are given in Table I.

TABLE I

EFFECT OF COMPOUND 1 ON THE PRECIPITATION OF ANTI-PN 34 RABBIT SERUM

Volume of diluted serum (μl)	*Weight of* 1 *(mg)*	*Volume of 0.01% S-34 (μl)*	*Weight of N (μg) in precipitate*
200	—	—	0.23
200	—	40	21.5
200	3	40	20.1

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*Capsular titer before dilution, 256; year of production, 1941. Generous gift from Dr. E. Lund, Staten Serum Institute, Copenhagen, Denmark.

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REACTION OF AZIDO SUGARS WITH ZINC DUST

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ABSTRACT

3-Azido-1,2:5,6-di-*O*-cyclohexylidene-3-deoxy-α-D-glucofuranose was easily reduced to the corresponding amino sugar with zinc dust in boiling aqueous *N*,*N*-dimethylformamide. 3-Azido-1,2-*O*-cyclohexylidene-3-deoxy-5,6-di-*O*-methylsulfonyl-α-D-glucofuranose was converted into 1,2-*O*-cyclohexylidene-3,6-dideoxy-3,6-epimino-5-*O*-methylsulfonyl-α-D-glucofuranose by treatment with sodium iodide and zinc dust in boiling aqueous *N*,*N*-dimethylformamide, whereas 3-azido-1,2-*O*-cyclohexylidene-3-deoxy-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose was converted into 3-amino-1,2-*O*-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-*xylo*-hexofuranose by the same treatment. The mechanisms of these two reactions were investigated; the present results suggest the neighboring participation of the azido group.

INTRODUCTION

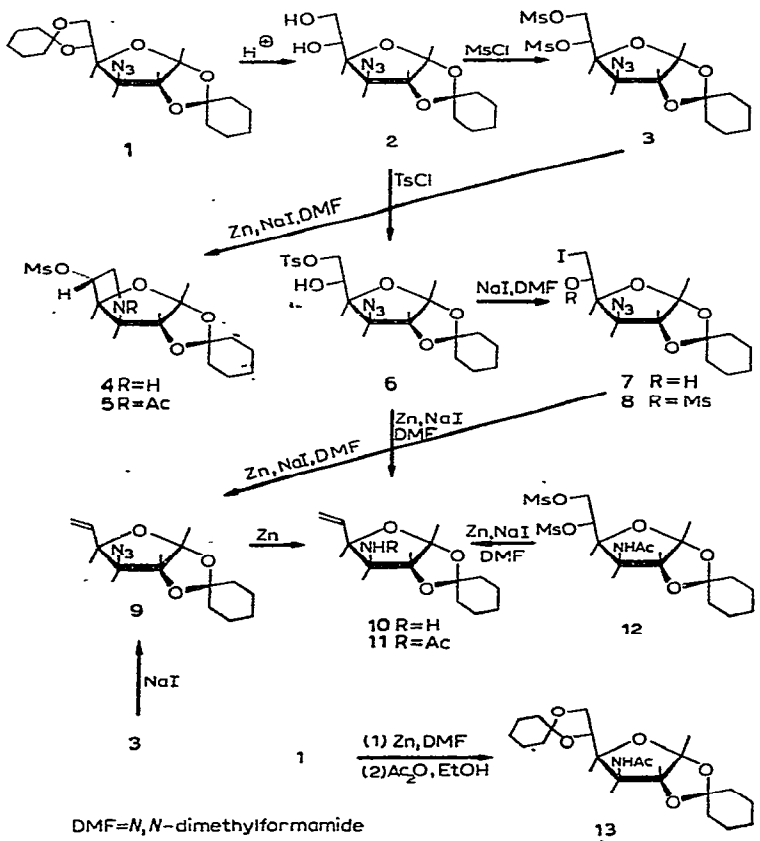
Carbohydrate azides are valuable intermediates for the synthesis of amino sugars. The azido group is usually converted into the corresponding amino group by catalytic reduction[1], or by treatment with lithium aluminum hydride[2] or sodium borohydride[3,4]. This paper describes the reduction of the azido group with zinc dust in boiling aqueous *N*,*N*-dimethylformamide, and the reaction of 3-azido-1,2-*O*-cyclohexylidene-3-deoxy-5,6-di-*O*-methylsulfonyl-α-D-glucofuranose (**3**) and 3-azido-1,2-*O*-cyclohexylidene-3-deoxy-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**6**) with sodium iodide and zinc dust in aqueous *N*,*N*-dimethylformamide[5]. This reaction is used for the preparation of unsaturated sugars. The occurrence of neighboring participation of the azido group[6,7] is postulated on the basis of the results obtained.

RESULTS AND DISCUSSION

It has been described previously[8] that the reduction of 3-azido-1,2:5,6-di-*O*-cyclohexylidene-3-deoxy-α-D-glucofuranose (**1**) with sodium borohydride required a time of 20–30 h. Subsequently we have found that **1** is easily reduced with zinc dust in boiling aqueous *N*,*N*-dimethylformamide within 45 min.

Partial hydrolysis of **1** with aqueous acetic acid gave in high yield 3-azido-1,2-

O-cyclohexylidene-3-deoxy-α-D-glucofuranose (**2**), which was methylsulfonylated in pyridine into 3-azido-1,2-*O*-cyclohexylidene-3-deoxy-5,6-di-*O*-methylsulfonyl-α-D-glucofuranose (**3**). Since treatment of 3-acetamido-1,2-*O*-cyclohexylidene-3-deoxy-5,6-di-*O*-methylsulfonyl-α-D-glucofuranose (**12**) with sodium iodide and zinc dust in boiling aqueous *N*,*N*-dimethylformamide had previously given 3-acetamido-1,2-*O*-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-*xylo*-hexofuranose (**11**)[8], compound **3** was similarly treated. It was, however, not converted into the expected product, but into a crystalline compound **4**, the i.r. spectrum of which did not show the presence of the azido group but of a methylsulfonyl group and a secondary amino group. Analytical data of **4** indicate a formula $C_{13}H_{21}NO_6S$. Acetylation of **4** with



acetic anhydride in ethanol gave the monoacetate **5**. Compound **4** was considered to be 1,2-*O*-cyclohexylidene-3,6-dideoxy-3,6-epimino-5-*O*-methylsulfonyl-α-D-glucofuranose (**4**) on the basis of its i.r. and n.m.r. spectra, and of its analytical data. The assignment of protons in the n.m.r. spectrum of **4** was made by the decoupling method, as shown in Fig. 1. The extra peak observed in the patterns for H-1 at a

sweep width of $9 \times 1/2$ is the result of a long-range coupling between H-1 and H-3.

On the basis of this result, it was expected that 3-azido-1,2-*O*-cyclohexylidene-3-deoxy-6-*O*-*p*-tolylsulfonyl-α-D-glucofuranose (**6**) would be converted into an epimino sugar by the same treatment, compound **6**, however, was not converted

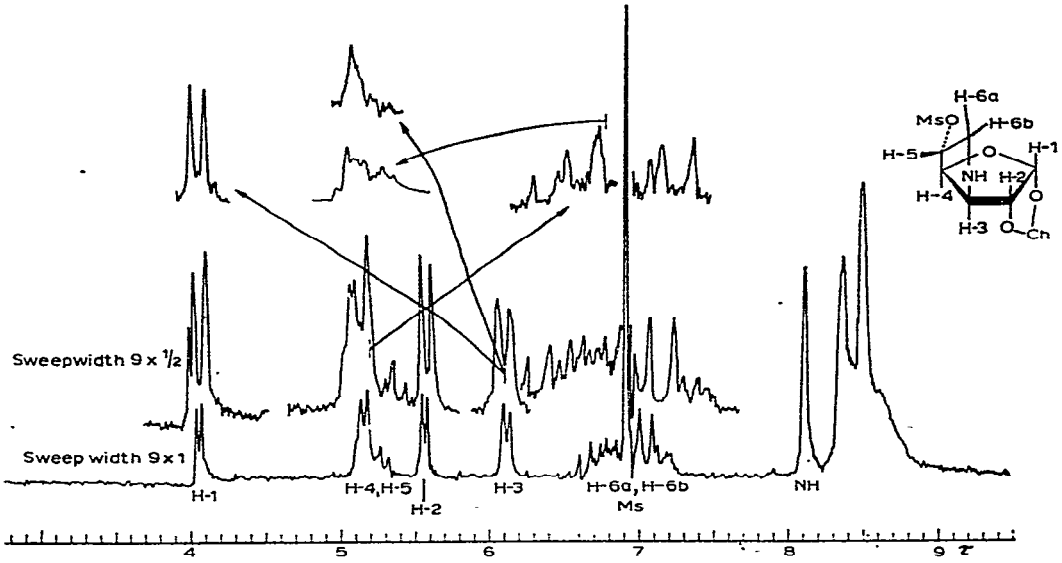


Fig. 1. N.m.r. spectrum of 1,2-*O*-cyclohexylidene-3,6-dideoxy-3,6-epimino-5-*O*-methylsulfonyl-α-D-glucofuranose (**4**) in deuteriochloroform at 100 MHz.

into an epimino sugar, but into the unexpected syrupy compound **10**. The i.r. spectrum of **10** showed the absence of azido, *p*-tolylsulfonyl, or hydroxyl group but the presence of a primary amino group. Compound **10** was acetylated with acetic anhydride in ethanol and gave a monoacetate identical, in all its physical and chemical properties, with **11** which had been obtained[8] from **12**. The assignment of protons in the n.m.r. spectrum of **10** is illustrated in Fig. 2, and the structure of 3-amino-1,2-*O*-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-*xylo*-hexofuranose was assigned to **10**.

It is of interest that the hydroxyl group at C-5 of **6** was eliminated, whereas the methylsulfonyl group at C-5 of **3** was resistant, since the latter group is more likely to be eliminated than the first one[9]. In an attempt to explain these results, **3** was treated with zinc dust in aqueous *N*,*N*-dimethylformamide for 1 h at 130° to give **4**.

Treatment of **3** with sodium iodide in aqueous *N*,*N*-dimethylformamide for 45 h at 130° gave a syrupy compound having the formula $C_{12}H_{17}N_3O_3$. The i.r. spectrum of this compound indicated the presence of an azido group and the absence of a methylsulfonyl group. The n.m.r. spectrum showed the presence of a terminal vinyl group. These results suggest the structure of the 3-azido-1,2-*O*-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-*xylo*-hexofuranose (**9**). It is of interest that compound **9** is stable even at 130°, although the azido group and the double bond are located in *cis* position to each other in the furanose ring.

The results of these two reactions suggest that both the formation of the imino sugar (by intramolecular S_N2 reaction of the methylsulfonyl group at O-6 with the nitrogen atom at C-3) and the double bond formation (by intermolecular

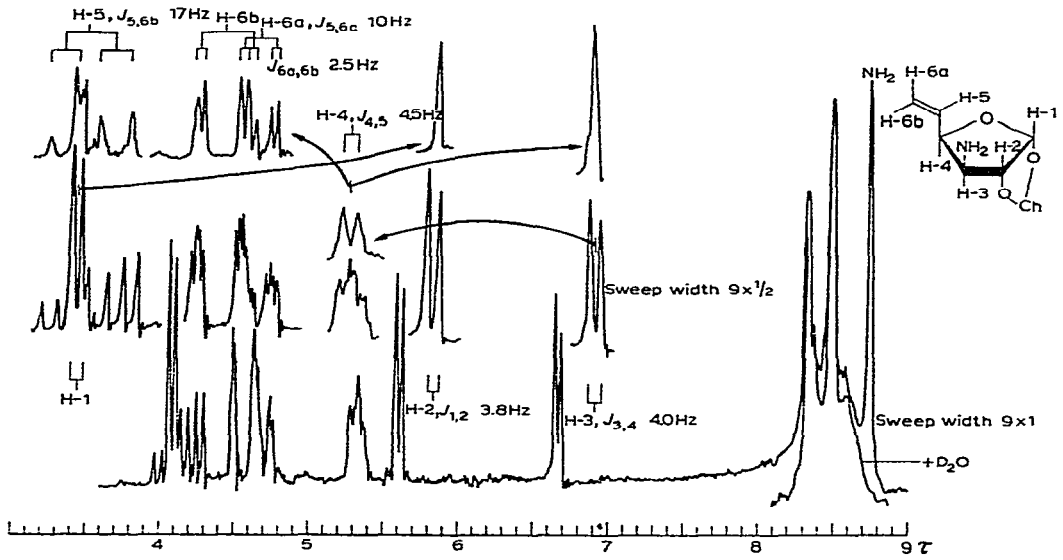


Fig. 2. N.m.r. spectrum of 3-amino-1,2-*O*-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-*xylo*-hexofuranose (**10**) in deuteriochloroform at 100 MHz.

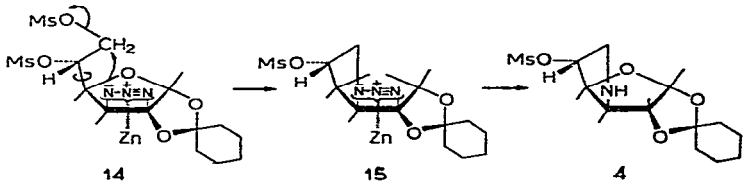
S_N2 reaction of the methylsulfonyl group at C-6 with the iodide ion, followed by its elimination) took place very easily, independently of each other; the intramolecular S_N2 reaction, however, proceeded more rapidly than the intermolecular S_N2 reaction in the presence of both zinc dust and sodium iodide.

When **6** was treated with sodium iodide in aqueous *N,N*-dimethylformamide for 1 h at 130°, t.l.c. of the reaction mixture indicated the disappearance of the starting material and the formation of three new materials. The main product, obtained as a syrupy substance by column chromatography, was shown to be 3-azido-1,2-*O*-cyclohexylidene-3,6-dideoxy-6-iodo-α-D-glucofuranose (**7**) on the basis of its i.r. spectrum and its elementary analysis. On the basis of the i.r. spectra, the other two by-products were shown to have retained the azido group and the hydroxyl group. Compound **10** was not obtained under these conditions. This result indicates that sodium iodide does not affect the azido group, nor does the double bond formation take place under these conditions.

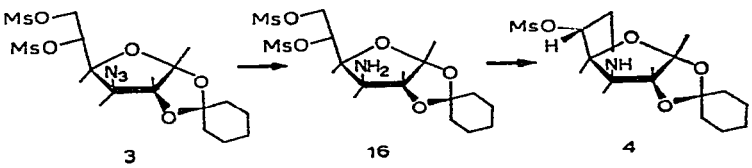
3-Azido-1,2-*O*-cyclohexylidene-3,6-dideoxy-6-iodo-5-*O*-methylsulfonyl-α-D-glucofuranose (**8**) was treated with sodium iodide and zinc dust in aqueous *N,N*-dimethylformamide at 130°. The reaction was followed by t.l.c. The amount of starting material (R_F 0.71) decreased gradually, and disappeared completely after 35 min. A compound giving a new spot (R_F 0.86) appeared after 3 min, reached a maximum amount after 12 min, and then disappeared completely after 35 min. The

reaction was terminated after 12 min, and the compound was obtained as a syrup by column chromatography. The structure of this reaction intermediate was shown to be **9** after study of its i.r. and n.m.r. spectra. A compound showing a third spot (R_F 0.04) appeared after 6 min, and its proportion increased gradually. After 35 min only this compound remained. Its structure was shown to be that of **10**. In this case, the intramolecular S_N2 reaction did not occur, and the double bond formation took place more rapidly than the reduction of the azido group. These results suggest that, during the treatment of **3** with sodium iodide and zinc dust in aqueous *N,N*-dimethylformamide, displacement of the methylsulfonyl group at C-6 by the iodide ion did not take place; if this reaction had occurred, formation of a 3,6-imino sugar would not be possible, and a 5,6-unsaturated sugar would result.

Although examples of the participation of a neighboring azido group[6,7] in replacement reactions in carbohydrates are rare, neighboring participation of the azido group or more probably of a metallo-azido complex was assumed in order to explain our results. If the azido group or the metallo-azido complex participates in the

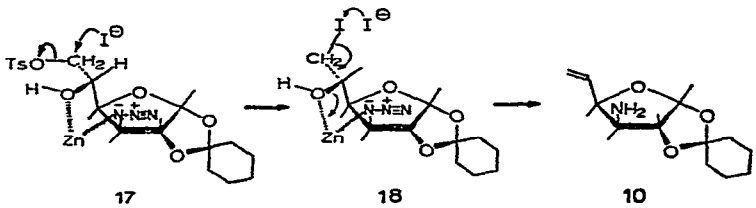


reaction, attack of these groups on the methylsulfonyl group at C-6 (intramolecular S_N2 reaction) (**14**) probably takes place more rapidly than that of the iodide ion (intermolecular S_N2 reaction). The resulting intermediate **15** is then reduced to **4**.



If, however, the epimino sugar is formed after the azido group has been reduced to an amino group, the displacement of the methylsulfonyl group at C-6 of **3** by an iodide ion must have been prevented during the reduction of the azido group, since the conversion of **3** into **9** proceeded very smoothly. But no explanation for this observation can be offered. On the other hand, in the treatment of **6** with sodium iodide and zinc dust in aqueous *N,N*-dimethylformamide, chelation between zinc and the free hydroxyl group at C-5 and the azido group at C-3 might occur (**17**), and the free rotation of the C-4–C-5 single bond would become restricted. If such is the case, the *p*-tolylsulfonyl group at C-6 is removed from the azido group at C-3, and the intramolecular S_N2 reaction by the azido group is overcome by the intermolecular

S_N2 reaction by the iodide ion (**18**). Thus, the elimination reaction and the reduction of the azido group can take place independently.



17 18 10

EXPERIMENTAL

All melting points are uncorrected. The optical rotations were measured with a Perkin–Elmer Model 141 photoelectric polarimeter (direct reading to ±0.002°) in a 1-dm tube. The infrared spectra were recorded with a Perkin–Elmer Model 521 grating infrared spectrophotometer or a Shimadzu IRS-27 Model D. The n.m.r. spectra were recorded with a Nihindenshi 4H-100 or a Nihondenshi C-60 n.m.r. spectrometer. The ascending t.l.c. were performed on Merck Kieselgel G with the solvent systems (A) benzene–ether (5:1), or (B) chloroform–methanol (95:5). The plates were sprayed with a mixture of methanol, sulfuric acid, and *p*-methoxybenzaldehyde (8.5:1:0.5), and then heated. The column chromatographies were performed on Wako Gel C-200.

3-Azido-1,2-O-cyclohexylidene-3-deoxy-α-D-glucofuranose (**2**). — 3-Azido-1,2:5,6-di-*O*-cyclohexylidene-3-deoxy-α-D-glucofuranose (**1**) (10 g) was dissolved in 75% acetic acid at 70°. The solution was kept for 30 min at this temperature, and then evaporated under reduced pressure at a bath temperature below 50°. The residue was dried by repeated evaporation in the presence of benzene (50 × 3 ml) to give crude crystals (9 g) which were recrystallized from hexane–ethyl acetate (10:1) into colorless crystals (7 g, 89.6%), m.p. 91–92°, $[\alpha]_D^{24}$ −55.0° (*c* 0.44, ethanol); i.r. data: ν_{max}^{KBr} 3180, 3260, 3320, 3405 (OH), and 2120 (N_3) cm^{-1}.

Anal. Calc. for $C_{12}H_{19}N_3O_5$: C, 50.52; H, 6.71; N, 14.73. Found: C, 50.52; H, 6.31; N, 14.79.

3-Azido-1,2-O-cyclohexylidene-3-deoxy-5,6-di-O-methylsulfonyl-α-D-glucofuranose (**3**). — To a solution of **2** (5.7 g) in dry pyridine (100 ml) was added at 0° methanesulfonyl chloride (10 g). The mixture was kept for 1 h at 0° and then for 3 h at room temperature. Ice–water (10 ml) was added and, after a few minutes, the mixture was poured into ice–water (300 ml), and then extracted with dichloromethane (5 × 50 ml). The dichloromethane solution was washed consecutively with ice-cooled 10% sulfuric acid (6 × 100 ml), saturated sodium hydrogen carbonate solution, and water. The organic layer was dried with sodium sulfate and evaporated. The syrupy residue (10 g) was chromatographed on a column and elution with chloroform–methanol (100:1) gave 8.0 g, (90%), $[\alpha]_D^{20}$ −36.2° (*c* 2.2, chloroform); i.r. data: ν_{max}^{film} 2110 ($-N_3$), 1360 and 1180 (CH_3SO_3-) cm^{-1}.

Anal. Calc. for $C_{14}H_{23}N_3O_9S_2$: C, 38.09; H, 5.25: N, 9.52; S, 14.53. Found: C, 38.33; H, 5.09; N, 9.31; S, 14.60.

1,2-O-Cyclohexylidene-3,6-dideoxy-3,6-epimino-5-O-methylsulfonyl-α-D-glucofuranose (**4**). — To a solution of **3** (10 g) in *N,N*-dimethylformamide (100 ml) was added a suspension of zinc dust (15 g) in an aqueous solution (10 ml) of sodium iodide (20 g). The mixture was heated for 1 h at reflux. After the reaction mixture was cooled, the zinc dust was filtered off and washed with *N,N*-dimethylformamide (10 ml). The combined filtrate and washings were poured into ice–water (300 ml) and extracted with dichloromethane (5 × 50 ml). The organic layer was washed with water (3 × 50 ml), dried with sodium sulfate, and evaporated under reduced pressure at a temperature below 50° to give crude crystals (6 g). Recrystallization from methanol gave colorless crystals (5.1 g, 68.4%), m.p. 166–167°; $[\alpha]_D^{24}$ +45.5° (*c* 0.66, chloroform); i.r. data: ν_{max}^{KBr} 3300 (-NH), 1350, and 1170 (CH_3SO_3-) cm^{-1}; n.m.r. data ($CDCl_3$): τ 8.45 (10 protons, protons in cyclohexane ring), τ 8.10 (1-proton singlet, -NH), τ 6.91 (3-proton singlet, CH_3SO_3-), τ 6.90 (multiplet, 2 protons, $H_{6a,6b}$), τ 6.10 (1-proton broad doublet, $J_{3,4}$ 2.7 Hz, H_{4-5}), τ 5.56 (1-proton doublet, $J_{1,2}$ 2.5 Hz, τ 5.14 (multiplet, 2-protons, H_{-4}, H_{-5}), τ 4.06 (1 proton, H_{-1}).

Anal. Calc. for $C_{13}H_{21}NO_6S$: C, 48.89; H, 6.63; N, 4.39; S, 10.04. Found: C, 49.00; H, 6.74; N, 4.40; S, 10.16.

3,6-Acetylepimino-1,2-O-cyclohexylidene-3,6-dideoxy-5-O-methylsulfonyl-α-D-glucofuranose (**5**). — Acetylation of **4** with acetic anhydride in ethanol gave a vitreous substance in almost quantitative yield, $[\alpha]_D^{21}$ −25.5° (*c* 0.71, ethanol); i.r. data: ν_{max}^{film} 1655 (-$NCOCH_3$), 1360, and 1179 (CH_3SO_3-) cm^{-1}.

Anal. Calc. for $C_{15}H_{23}NO_7S$: C, 49.85; H, 6.41; N, 3.88; S, 8.78. Found: C, 49.93; H, 6.64; N, 3.88; S, 8.87.

3-Azido-1,2-O-cyclohexylidene-3-deoxy-6-O-p-tolylsulfonyl-α-D-glucofuranose (**6**). — To a solution of **2** (3.4 g) in dry pyridine (200 ml) cooled to −10° was added slowly *p*-tolylsulfonyl chloride (2.7 g). The mixture was stirred for 1 h at −10°, and then for 10 h at room temperature, poured into ice–water (200 ml), and extracted with dichloromethane (5 × 100 ml). The organic layer was washed consecutively with ice-cooled 10% sulfuric acid (12 × 100 ml), saturated sodium hydrogen carbonate solution, and water, dried with sodium sulfate, and evaporated to a brown syrup (5 g), which was chromatographed on a column. Elution with chloroform–methanol (95:5) gave 2.9 g (55%), $[\alpha]_D^{25}$ −16.9° (*c* 0.73, ethanol); i.r. data: ν_{max}^{film} 3500 (-OH), 2110 ($-N_3$), 1603 (C_6H_5-), 1373, and 1180 (*p*-tolylsulfonyl) cm^{-1}.

Anal. Calc. for $C_{19}H_{25}N_3O_7S$: C, 51.92; H, 5.73; N, 9.56; S, 7.30. Found: C, 52.16; H, 5.46; N, 9.70; S, 7.56.

3-Azido-1,2-O-cyclohexylidene-3,6-dideoxy-6-iodo-α-D-glucofuranose (**7**). — To a solution of **6** (1 g) in *N,N*-dimethylformamide (20 ml) was added an aqueous solution of sodium iodide (2 g in 2 ml of water). The mixture was heated for 1 h at reflux, and then cooled, and poured into ice–water (100 ml). After extraction with dichloromethane (4 × 50 ml), the combined organic phases were washed with 0.1M sodium thiosulfate solution (2 × 50 ml) and then with water (2 × 50 ml), dried with sodium

sulfate, and evaporated to give a syrup (500 mg), which was chromatographed on a column. Elution with benzene–ether (20:1) gave two fractions, the first one containing 7 (380 mg, 42%), $[\alpha]_D^{23}$ −20.7° (*c* 3.2, ethanol); i.r. data: ν_{max}^{film} 3490 (-OH), and 2110 ($-N_3$) cm^{-1}.

Anal. Calc. for $C_{12}H_{17}IN_3O_4$: C, 36.47; H, 4.59; I, 32.11; N, 10.63. Found: C, 36.95; H, 4.68; I, 31.65; N, 10.75.

The second fraction contained two unknown substances (110 mg) as observed by t.l.c. in solvent system A. I.r. data of the mixture: ν_{max}^{film} 3460 (-OH), 2110 ($-N_3$), and 1711 (-CO) cm^{-1}.

3-Azido-1,2-O-cyclohexylidene-3,6-dideoxy-6-iodo-5-O-methylsulfonyl-α-D-glucofuranose (**8**). — To a solution of 7 (1.5 g) in dry pyridine (10 ml) cooled to 0° was added methylsulfonyl chloride (700 mg). The mixture was kept for 3 h below 5° and for 2 h at room temperature, poured into ice–water (20 ml), and extracted with dichloromethane (5 × 10 ml). The extract was washed consecutively with cold 100 mM sulfuric acid (3 × 20 ml), saturated sodium hydrogen carbonate solution, and water dried with sodium sulfate, and evaporated under reduced pressure at a temperature below 40°. The residual syrup was chromatographed on a column to give 930 mg (56.8%), $[\alpha]_D^{22}$ −64.7° (*c* 3.3, ethanol); i.r. data: ν_{max}^{film} 2110 ($-N_3$), 1350, and 1175 (CH_3SO_3-) cm^{-1}.

Anal. Calc. for $C_{13}H_{20}IN_3O_6S$: C, 32.99; H, 4.26; N, 8.88. Found: C, 33.21; H, 4.45; N, 8.83.

3-Azido-1,2-O-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-xylo-hexofuranose (**9**). — To a solution of 3 (500 mg) in *N,N*-dimethylformamide (10 ml) was added a solution of sodium iodide (500 mg) in water (1 ml). The mixture was heated for 1 h at reflux. T.l.c. in solvent *A* showed the formation of only one product. The solution was cooled and poured into ice–water (50 ml), and extracted with ether (3 × 50 ml). The ether solution was washed with 100 mM sodium thiosulfate solution (2 × 30 ml) and water (2 × 25 ml), dried with sodium sulfate, and evaporated to a brown syrup (250 mg). This was purified by column chromatography (100:1 benzene–ether) to give a pure syrup (230 mg, 80.1%), $[\alpha]_D^{21}$ −23.4° (*c* 1.7, ethanol); i.r. data, ν_{max}^{film} 2110 ($-N_3$) cm^{-1}; n.m.r. data ($CDCl_3$): τ 4.08 (1-proton doublet, $J_{1,2}$ 4 Hz H-1), τ 3.90-4.76 (3-proton multiplet, H-5,6,6'), τ 5.27 (1-proton multiplet, H-4), τ 5.40 (1-proton doublet, $J_{1,2}$ 4 Hz, H-2), τ 6.15 (1-proton doublet, $J_{3,4}$ 4 Hz, H-3), τ 8.20–8.25 (10-proton, protons in cyclohexane ring).

Anal. Calc. for $C_{12}H_{17}N_3O_3$: C, 57.35; H, 6.82; N, 16.72. Found: C, 57.57; H, 6.52; N, 16.80.

3-Amino-1,2-O-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-D-xylo-hexofuranose (**10**). (*i*). — To a solution of monoacetate 6 (1.016 g) in *N,N*-dimethylformamide (20 ml) was added zinc dust (1.5 g) and a solution of sodium iodide (2 g) in water (2 ml). The mixture was heated for 1 h at reflux and cooled, and the zinc dust was filtered off and washed with *N,N*-dimethylformamide (2 × 10 ml). The filtrate and washings were poured into ice-water (100 ml) and extracted with dichloromethane (5 × 25 ml). The extract was dried with sodium sulfate and evaporated.

Column chromatography of the residual syrup (460 mg) gave a very hygroscopic syrup (300 mg, 57.6%), i.r. data: ν_{max}^{film} 3340, and 3280 (-NH_2) cm^{-1}.

(*ii*). — To a solution of **8** (230 mg) dissolved in *N*,*N*-dimethylformamide (5 ml), zinc dust (300 mg) and a solution of sodium iodide (300 mg in 0.5 ml of water) were added. The mixture was heated for 35 min at reflux, and then cooled, and the zinc dust was filtered off and washed with *N*,*N*-dimethylformamide (2 ml). The filtrate and washings were combined and treated as previously described to give a syrup (150 mg) which was purified by column chromatography (109 mg, 100%).

In one experiment, the reaction was stopped after a reflux of 12 min, and the reaction mixture was examined by t.l.c. in benzene–ether (5:1) after treatment as previously described. It showed a considerable amount of unchanged starting material and the formation of two products. The crude syrup was chromatographed on a column, and elution with benzene–ether (5:1) gave three fractions. The first one was a syrup identified as **9** by i.r. and n.m.r. spectra, and by t.l.c. The second fraction, also a syrup, was identified as **10** by i.r. and n.m.r. spectra, and t.l.c. The third fraction was the starting material.

*3-Acetamido-1,2-O-cyclohexylidene-5,6-didehydro-3,5,6-trideoxy-α-*D-xylo-*hexofuranose* (**11**). — To a solution of **10** (100 mg) in ethanol (5 ml) was added acetic anhydride (1 ml). The mixture was kept for 10 min at 50°, and then evaporated to dryness to give colorless crystals, which were recrystallized from hexane–methanol (10:1) (110 mg, 100%), m.p. 143°, $[\alpha]_D^{24}$ +31.6° (*c* 1.0, ethanol).

Anal. Calc. for $C_{14}H_{21}NO_4$: C, 62.90; H, 7.92; N, 5.24. Found: C, 62.94; H, 7.65; N, 5.43.

*3-Acetamido-1,2:5,6-di-O-cyclohexylidene-3-deoxy-α-*D-*glucofuranose* (**13**). — To a solution of **1** (500 mg) in 90% aqueous *N*,*N*-dimethylformamide (11 ml) was added zinc dust (750 mg). The mixture was heated for 1.5 h at reflux, cooled, and then poured into ice–water (100 ml) and extracted with dichloromethane (20 × 5 ml). The combined extracts were washed with water, dried with sodium sulfate, and evaporated under reduced pressure to give a syrup. This was dissolved in ethanol (20 ml), and acetic anhydride (2.5 ml) was added. The mixture was kept for 30 min at 50° and then evaporated to give a crude syrup, which was chromatographed on a column. Elution with chloroform–methanol (95:5) gave a glass (490 mg, 95%), $[\alpha]_D^{22}$ +0.5° (*c* 1.6, ethanol).

Anal. Calc. for $C_{20}H_{31}NO_6$: C, 62.97; H, 8.19; N, 3.67. Found: C, 62.94; H, 8.10; N, 3.57.

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PROTEIN–CARBOHYDRATE INTERACTION

PART XXI. THE INTERACTION OF CONCANAVALIN A WITH D-FRUCTANS*

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ABSTRACT

The immunochemical techniques of quantitative precipitation and hapten inhibition have been employed in a study of the interaction of concanavalin A with several levans of bacterial and plant origin. Of the polysaccharides that form a precipitate with concanavalin A (dextrans, D-mannans, glycogens, amylopectins, and levans), levans appear to be the least reactive.

Hapten-inhibition studies were conducted with a wide variety of compounds containing a furanoid ring. As a generalization, it may now be stated that all sugars containing the 1,4-anhydro-D-arabinitol moiety can bind to the active sites of concanavalin A. Because evidence from this laboratory suggests that the interaction of concanavalin A with reactive polysaccharides occurs at the same binding sites of the protein, reaction with levans may be rationalized on the basis of common configurational features. The disposition of the hydroxyl groups on C-3, C-4, and C-6 (or C-1, C-3, and C-4) of the β-D-fructofuranosyl moiety, which is the residue that occurs at chain ends of levans, is similar to that on C-3, C-4, and C-6 of the α-D-glucopyranosyl moiety, the sugar residue found at the chain ends of α-D-glucans.

INTRODUCTION

In earlier studies, concanavalin A was shown to react only with polysaccharides containing D-glucose and D-mannose[1–3]. The first demonstration of the interaction of concanavalin A with levans was reported by Goldstein and So[4,5], who used the Ouchterlony, double-diffusion technique. Recently, it was reported[6] that the levan from *L. mesenteroides* strain C also reacts to form a precipitate with concanavalin A. Levans are high molecular-weight, branched-chain D-fructans, elaborated in the growth media of certain micro-organisms, in which the β-D-fructofuranosyl residues are joined via (2→6) glycosidic linkages, with branch points originating from the

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hydroxymethyl groups of every 9–12 D-fructofuranosyl residues. Levans of plant origin are also known[7].

In the present study, the immunochemical techniques of quantitative precipitation[8] and hapten inhibition[9,10] (see Kabat[11] for an extended discussion of these techniques) have been employed in an examination of levan–concanavalin A interaction. To establish a basis for this interaction, a variety of sugars containing D-fructofuranosyl residues was examined for their capacity to inhibit the concanavalin A–polysaccharide interaction.

MATERIALS AND METHODS

Aerobacter levanicum No. 15552 was obtained from the American Type Culture Collection. *Bacillus megatherium* KM was a gift from Dr. J. M. Merrick. Dextran NRRL B-1355-S and the levan from *Bacillus subtilis* were generous gifts from Drs. Allene R. Jeanes and D. Feingold, respectively. A D-gluco-D-fructan from the Hawaiian Ti plant was obtained from Dr. B. Lewis. Planteose was a gift from Dr. D. French; the methyl ethers of D-fructose were provided by Dr. Elizabeth E. Percival; allosucrose was a gift from Dr. D. Feingold; the 1,4-anhydropentitols and 2,5-anhydro-L-iditol and 2,5-anhydro-D-mannitol were provided by Dr. R. Barker; 2,5-anhydro-D-glucitol was presented by Dr. J. W. LeMaistre; 1,5-anhydro-D-glucitol and 1,5-anhydro-D-mannitol were the gift of Dr. N. K. Richtmyer; 1-kestose and nystose were given to us by Dr. W. W. Binkley; Dr. C. T. Bishop provided the methyl pentofuranosides; and Dr. B. Lewis, di-D-fructose 1,2′:2,1′-dianhydride. Inulobiose and inulotriose were isolated from a partial, acid-hydrolyzate of inulin (Mann Research Laboratories, Inc., New York) by descending paper-chromatography with butyl alcohol–ethanol–water [4:1:5 (v/v), upper phase] under the conditions of Pazur and Gordon[12]. Planteobiose was isolated from planteose according to the procedure of French *et al.*[13]. Mr. Gordon Hassing of this laboratory provided samples of the four methyl glycosides of D-fructose.

Concanavalin A was obtained in a highly purified state by the Sephadex method of Agrawal and Goldstein[14].

Cross-linked, levan gel was provided by Dr. E. A. Kabat.

Growth of bacteria. — Stock cultures of *A. levanicum* were maintained on nutrient-agar slants (Difco Laboratories, Detroit, Michigan), and grown in nutrient broth (Difco Laboratories) containing sucrose, as described by Hestrin *et al.*[15]. Sucrose solutions were sterilized by filtration through a bacterial, ultra-fine filter (E. H. Sargent and Co.).

Stock cultures of *Bacillus megatherium* KM were maintained on 1% yeast extract in 1.5% agar. Cells were grown in the presence of sucrose. The growth medium (personal communication, Dr. J. M. Merrick) contained 266.6 ml of 0.1M Na_2HPO_4, 133.3 ml of 0.1M KH_2PO_4, 3.0 g of NaCl, 1.0 g of NH_4Cl, 0.1 g of Na_2SO_4, 0.1 g of $MgCl_2 \cdot 6\,H_2O$, 0.01 g of $MnCl_2 \cdot 4\,H_2O$, and 0.1 g casamino acid (vitamin-free) per liter of solution.

Isolation of levan. — After growth for 24 h at 30°, cells were harvested by centrifugation at 8,000 r.p.m. for one h in a Sorvall RC-2 centrifuge equipped with a GSA head. Levan was precipitated from the supernatant solution by the addition of approximately three volumes of 95% ethanol; addition of a few drops of 1% calcium chloride solution facilitated flocculation[15]. The gummy precipitate that separated was triturated with absolute methanol. Purification was achieved by dissolution in warm water, filtration, reprecipitation with ethanol, and trituration with methanol. Deproteinization with one-third of the volume of 3:1 (v/v) chloroform–1-octanol was effected by the Sevag procedure[16].

Complete hydrolysis, with acid, of levans isolated from the growth media of *A. levanicum* and *B. megatherium* gave fructose as the sole constituent monosaccharide on paper-chromatographic analysis. Hydrolysis was conducted by heating a solution of the levan in 5% aqueous oxalic acid in a sealed tube for one h in a boiling-water bath. The solution was made neutral with solid calcium carbonate, the suspension was filtered, and the filtrate was concentrated *in vacuo* at 40°. Paper chromatography was performed by the descending technique, with butyl alcohol–ethanol–water [4:1:5 (v/v), upper phase] as the irrigating solvent. After being dried, paper chromatograms were sprayed with *p*-anisidine[17].

Quantitative precipitation. — Studies on quantitative precipitation were performed as described by So and Goldstein[8]. Increasing amounts of polysaccharide were added to a series of tubes containing a constant amount of concanavalin A. A typical incubation mixture consisted of the following components: concanavalin A in M NaCl (0.1 ml), 4.1M NaCl (0.22 ml), 0.1M phosphate buffer (pH 7.2, 0.18 ml), and polysaccharide in water (0.5 ml).

The amount of protein nitrogen in the precipitates was determined as previously described[8], by ninhydrin analysis of the washed precipitates, which had been digested with 3.5M sulfuric acid.

Carbohydrate analysis of precipitates. — The carbohydrate composition of concanavalin A–levan precipitates was determined by the phenol–sulfuric acid method[18], performed directly on the protein–polysaccharide precipitates, as previously described[8]. The total amount of polysaccharide in the precipitate was determined by reference to a standard curve for D-fructose, the conversion to give the value for polysaccharide being made by multiplying by 0.9.

The concentration of all solutions of polysaccharide, as well as those of sugars employed in hapten inhibition, was determined by the phenol–sulfuric acid method[18].

Hapten inhibition. — Experiments on hapten inhibition were conducted by determining the extent to which various carbohydrates of low molecular weight inhibited concanavalin A–dextran precipitation[10]. A typical, incubation mixture employed in these studies contained the following components: concanavalin A (300 μg) in M NaCl (0.1 ml), 4.1M NaCl (0.22 ml), 0.1M phosphate buffer (pH 7.2, 0.18 ml), dextran B-1355-S (200 μg, 0.1 ml), and inhibitor in water (0.4 ml). When the concanavalin A–levan (*A. levanicum*) system was employed in hapten-inhibition studies, 100 μg of levan was used in quantitative precipitation, and analysis for protein

nitrogen was conducted as described earlier. Incubation mixtures containing no inhibitor were used simultaneously, as controls, in all experiments on inhibition. Percentage inhibition was calculated as being equal to the difference between the amount of nitrogen precipitated in the absence and presence of inhibitor, divided by that in the absence of inhibitor, and multiplied by 100.

RESULTS

Quantitative precipitation studies. — The quantitative precipitation curves of concanavalin A with the levans from *A. levanicum*, *B. megatherium*, and *B. subtilis*, obtained by plotting μg of nitrogen found in the precipitate (by ninhydrin analysis) against mg of levan added, are shown in Figs. 1, 2, and 3, respectively. The carbohydrate contents of the concanavalin A–levan precipitates at each stage of the precipitation curves are also shown.

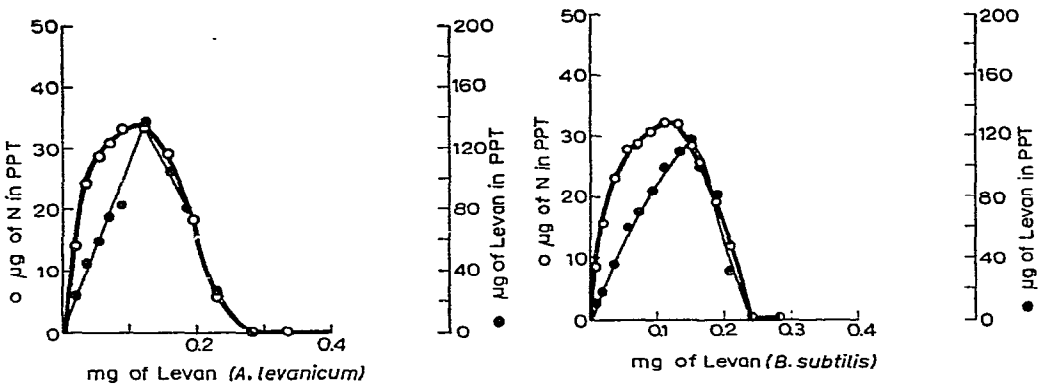


Fig. 1. Quantitative precipitation curve of *A. levanicum* levan with concanavalin A (48 μg of nitrogen). The total amount of levan precipitated is also shown.

Fig. 2. Quantitative precipitation curve of a levan from *B. subtilis* with concanavalin A (42 μg of nitrogen). The total amount of levan precipitated is also shown.

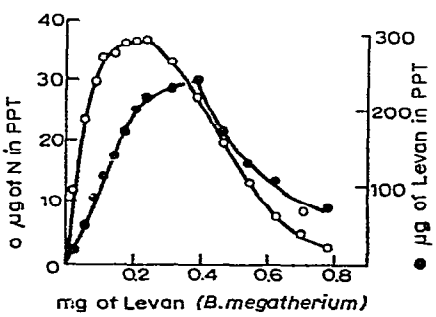


Fig. 3. Quantitative precipitation curve of a levan from *B. megatherium* with concanavalin A (49 μg of nitrogen). The amount of carbohydrate precipitated is also shown.

In contrast to dextran[8] B-1355-S and a variety of α-D-mannans[19], which precipitate 95–98% of the total concanavalin A added, these levans precipitated, at the region of maximum precipitation, only about 70–78% of the total concanavalin A added. In the region of an excess of concanavalin up to the point of maximum precipitation, virtually all of the levan added was found to be in the precipitates.

TABLE I

INHIBITION OF CONCANAVALIN A–POLYSACCHARIDE PRECIPITATION BY MONO- AND OLIGO-SACCHARIDES AND SOME OF THEIR DERIVATIVES

Saccharide	*μMoles giving 50% inhibition in the dextran–concanavalin A system*[a]	*μMoles giving 50% inhibition in the levan–concanavalin A system*[b]
Methyl α-D-mannopyranoside	0.6	0.087
Methyl β-D-fructopyranoside	0.85	0.12
Methyl α-D-glucopyranoside	2.5	0.36
Methyl α-L-sorbopyranoside	3.1	
D-Mannose	4.4	
Methyl β-D-fructofuranoside	5.7	0.94
D-Fructose	9.3	1.5
Methyl α-D-fructofuranoside	16	2.3
D-Glucose	21	3.5
Methyl α-D-fructopyranoside	107 (40%)[c]	
D-Allose	97 (4%)	
D-Galactose	104 (8%)	
Melezitose	0.71	
Leucrose	0.85	
Inulotriose	4.6	
Nystose	4.9	
Turanose	5.6	
Levanbiose	6.9	
1-Kestose	7.8	
Inulobiose	9.6	
Di-D-fructose 1,2′:2,1′-dianhydride	13.5	
Sucrose	23	4.0
Planteobiose	35 (25%)	
Planteose	34 (9%)	
Raffinose	100 (23%)	
Lactulose	100 (4%)	

[a]Each tube contained concanavalin A (300 μg), dextran NRRL-B-1355-S (200 μg), and inhibitor as noted, in a total volume of 1.0 ml. [b]Each tube contained concanavalin A (300 μg), *A. levanicum* levan (100 μg), and inhibitor as noted, in a total volume of 1.0 ml. [c]Numbers in parentheses refer to percentage inhibition given by the μmoles of saccharide noted.

Fig. 4 presents the curve for precipitation of concanavalin A and the D-gluco-D-fructan from the Hawaiian Ti plant. The interaction is relatively weak, as, of the 46 μg, only about 19 μg (41%) of concanavalin A nitrogen was precipitated at the point of maximum precipitation.

Hapten-inhibition studies. — Unless specified otherwise, hapten-inhibition experiments were conducted with the concanavalin A–dextran (B-1355-S) system[10].

The inhibition caused by several monosaccharides and their derivatives is shown in Table I. As in previous studies, D-mannose was found to be a more potent inhibitor than D-fructose, which, in turn, was superior to D-glucose; methyl α-D-glucopyranoside was approximately 8.4 times as potent as D-glucose.

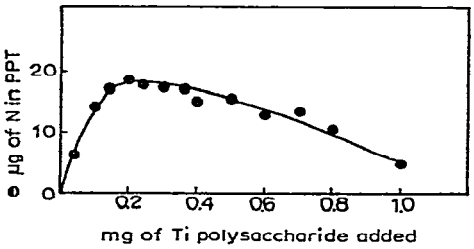


Fig. 4. Quantitative precipitation curve of the D-gluco-D-fructan from the Hawaiian Ti plant, with concanavalin A (46 μg of nitrogen).

Of the four methyl glycosides of D-fructose, methyl α-D-fructopyranoside was a relatively poor inhibitor (40% at 107 μmoles) compared with the three other methyl fructosides, the degree of potency decreasing in the order: methyl β-D-fructopyranoside > methyl β-D-fructofuranoside > methyl α-D-fructofuranoside. Methyl α-L-sorbopyranoside (3.1 μmoles for 50% inhibition) was a less potent inhibitor than methyl β-D-fructopyranoside.

A large number of anhydropentitols and anhydrohexitols were also investigated for their capacity to inhibit the concanavalin A–dextran system; these are listed in Table II. Of the anhydroalditols containing a tetrahydrofuran ring, only those containing the 1,4-anhydro-D-arabinitol ring-system were found to be inhibitors of

TABLE II

INHIBITION OF PRECIPITATION BY ANHYDRO ALDITOLS

Sugar	*μMoles*	*Inhibition, %*
1,4-Anhydro-DL-allitol	57	2.2
	105	7.1
1,4-Anhydro-D-altritol	56	5.2
1,4-Anhydro-D-arabinitol	100	44
1,4-Anhydro-D-glucitol	50	3.5
1,5-Anhydro-D-glucitol	14	50
2,5-Anhydro-D-glucitol	19	70
2,5-Anhydro-L-iditol	55	1.8
1,4-Anhydro-D-lyxitol	52	0
1,4-Anhydro-D-mannitol	49	4.8
1,5-Anhydro-D-mannitol	4.4	50
2,5-Anhydro-D-mannitol	19	50
1,4-Anhydro-DL-ribitol	52	0
	100	4
1,4-Anhydro-DL-xylitol	47	3.8
	103	6.1

concanavalin A–polysaccharide precipitation. Thus, among the 1,4-anhydropentitols, only 1,4-anhydro-D-arabinitol was an inhibitor of any consequence.

Both 2,5-anhydro-D-mannitol (19 μmoles for 50% inhibition) and 2,5-anhydro-D-glucitol (70 μmoles for 50% inhibition) possess the requisite 1,4-anhydro-D-arabinitol ring-system, and hence behave as inhibitors. 1,5-Anhydro-D-mannitol and 1,5-anhydro-D-glucitol are considerably better inhibitors than their corresponding 2,5-anhydro derivatives. Structural correlations of all of these anhydroalditols, together with a rationale for their reactivity in the concanavalin A system, are presented in the Discussion.

Inhibition analysis of many of the D-pentofuranosides, together with that for several of the corresponding pyranosides, is presented in Table III, from which is evident that only methyl α- and β-D-arabinofuranoside bind to the active sites of

TABLE III

INHIBITION OF PRECIPITATION BY PENTOSES AND METHYL PENTOSIDES

Sugar	*μMoles*	*Inhibition, %*
D-Arabinose	110	24
L-Arabinose	96	6
Methyl α-D-arabinofuranoside	51	50
Methyl β-D-arabinofuranoside	60	50
Methyl β-L-arabinopyranoside	52	0.2
Methyl α-D-lyxofuranoside	60	4.3
Methyl β-D-ribofuranoside	54	0.5
Methyl α-D-xylofuranoside	52	0.2
Methyl α-D-xylopyranoside	64	4.6
Methyl β-D-xylopyranoside	58	3.1
D-Ribose	107	8
D-Xylose	106	5

the protein molecule. Furthermore, of the four D-aldopentoses, only D-arabinose (but not L-arabinose) inhibited the concanavalin A–dextran system. It may be noted that D-ribose, reported by Doyle *et al.*[20] to be somewhat active in the concanavalin A system, is without activity at a level of 107 μmoles.

Several oligosaccharides containing D-fructofuranosyl residues were also tested for their capacity to inhibit concanavalin A–polysaccharide interaction (see Table I). In the series of sucrose, 1-kestose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*], nystose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*], the percentage inhibition increased in that order. Levanbiose (6.9 μmoles for 50% inhibition) is a slightly better inhibitor than inulobiose (9.6 μmoles for 50% inhibition), and inulotriose (4.6 μmoles for 50% inhibition) is superior to inulobiose. A difructosan composed of two β-D-fructofuranosyl residues united by 1,2′:2,1′-anhydride linkages gave 50% inhibition at a level of 13.5 μmoles. Leucrose [α-D-G*p*-(1→5)-D-Fru*p*; 0.85 μmole for 50% inhibition] and turanose [α-D-G*p*-(1→3)-D-Fru] (5.6 μmoles for 50% inhibition) were found to be better inhibitors than sucrose (23 μmoles for 50% inhibition).

Melezitose [α-D-Glc*p*-(1→3)-β-D-Fru*f*-(2↔1)-α-D-Glc*p*; 0.71 μmole for 50% inhibition] is almost as effective an inhibitor as methyl α-D-mannopyranoside (0.64 μmole for 50% inhibition); the latter is one of the most potent inhibitors of concanavalin A–polysaccharide precipitation thus far examined. With the exception of planteobiose [α-D-Gal*p*-(1→6)-D-Fru*f*; 35 μmoles for 25% inhibition], the remaining oligosaccharides containing D-fructofuranosyl moieties (raffinose, gentianose, planteose, and lactulose) were essentially ineffective as inhibitors of precipitation.

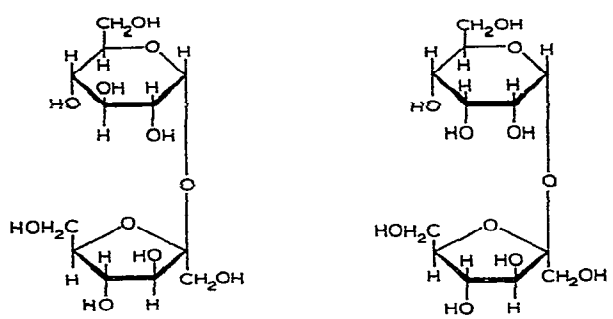
Several methyl ethers of D-fructose were examined for their inhibitory activity in the concanavalin A–polysaccharide reaction. It was found that modification of the 3- or 4-hydroxyl groups of D-fructose gave compounds that still produced significant inhibition of precipitation, 4-*O*-methyl-D-fructose (19 μmoles for 93% inhibition) being a more potent inhibitor than 3-*O*-methyl-D-fructose (19 μmoles for 30% inhibition) (see Table IV). The di-, tri-, and tetra-methyl ethers of D-fructose that were tested were found to be noninhibitory.

TABLE IV

INHIBITION OF PRECIPITATION BY METHYL ETHERS OF D-FRUCTOSE

Ether of D-*fructose*	*μMoles*	*Inhibition, %*
3-Methyl	19	30
4-Methyl	19	93
3,4-Dimethyl	87	0
1,3,4-Trimethyl	20	2
1,3,4,6-Tetramethyl	32	7

Inhibition data obtained with sucrose and allosucrose [α-D-All*p*-(1↔2)-β-D-Fru*f*] demonstrated that, in sucrose, it is the D-glucosyl moiety that is principally involved in binding, despite the presence in the D-fructosyl moiety of the determinants necessary for interaction with concanavalin A. The difference in structure between sucrose and allosucrose lies only in the disposition of the 3-hydroxyl group of the hexopyranosyl moiety (see Fig. 5). At a level of 52 μmoles, allosucrose was a noninhibitor, whereas



Sucrose

23 μmoles; 50% inhibition

Allosucrose

52 μmoles; 0% inhibition

Fig. 5. Inhibition of precipitation by sucrose and allosucrose.

at 23 μmoles, sucrose gave 50% inhibition. It should be noted that D-allose is a noninhibitor of precipitation (4% at 97 μmoles).

Inhibition studies were conducted with the concanavalin A–levan (*A. levanicum*) system, and it was found that the relative order of activity of the different inhibitors examined paralleled exactly that shown for the concanavalin A–dextran (B-1355-S) system[10]; however, smaller proportions of the various sugars were required for the same percentage inhibition (Table I).

Chromatography on cross-linked, levan gel. — When a solution of protein from jack-bean meal, precipitated between 0.3 and 0.8 saturation with respect to $(NH_4)_2SO_4$, was passed through a small column of cross-linked, levan gel, a chromatographic pattern similar to that obtained with the Sephadexes was obtained[14]. All of the proteins unreactive with the levan matrix appeared with the void volume (peak No. 1). On addition of a solution of D-glucose (0.1M) to the column, the active protein was eluted (peak No. 2). About 60% of the total protein applied to the column was recovered in the first peak. The remaining protein that was displaced by D-glucose was active, the activity being determined by quantitative precipitation[8] with dextran B-1355-S after extensive dialysis to remove D-glucose. Peak No. 1 gave no precipitate with dextran B-1355-S.

D-Galactose, D-xylose, melibiose, cellobiose, and lactose did not displace the protein from the column, whereas either D-fructose or D-glucose, at a concentration of 100 mM, did. When a 10 mM solution of D-glucose was employed as eluant, the adsorbed protein was incompletely displaced.

Concanavalin A isolated by the Sephadex-adsorption method was specifically bound to cross-linked, levan gel, and was displaced as a single peak by D-glucose. No protein was eluted with the void volume of the column.

DISCUSSION

Bacterial levans containing multiple, terminal β-D-fructofuranosyl residues have been shown herein to be precipitants of concanavalin A[4-6,21], maximum precipitations occurring at pH 6.0–7.2, which is the same pH range as observed with dextrans[8] and D-mannans[19]. Concanavalin A–levan (*A. levanicum*) precipitates, however, are more soluble (3.5 μg of N/ml)[22] compared with concanavalin A–dextran (1.5 μg of N/ml)[8] or concanavalin A–D-mannan (1.0 μg of N/ml)[19] precipitates. As with results obtained with other polysaccharides, precipitation curves of D-fructans with concanavalin A demonstrated, as in the case of antibody–antigen precipitation, the presence of three zones of precipitation; these are a zone of (*1*) concanavalin A excess, (*2*) "equivalence", and (*3*) levan excess. In the region of excess of concanavalin A, all of the levan added is completely precipitated. However, in the region of "equivalence" (or maximum precipitation of protein), incomplete precipitation of both the protein and the polysaccharide was observed. With *A. levanicum* levan as the precipitant, for example, only 72% of the total protein-nitrogen added was found in the precipitate, compared with a value of 95–98% for dextrans and D-mannans. The

levans from *B. subtilis* and *B. megatherium* precipitated only 74 and 78%, respectively, of the total concanavalin A at the region of maximum precipitation. This result is believed to be attributable to the low reactivity of this type of polysaccharide and to the greater solubility of concanavalin A–levan precipitates. This conclusion will become more evident from an evaluation of the inhibition experiments described later.

As an example of the interaction of a plant D-fructan with concanavalin A, we investigated the reaction with the D-gluco-D-fructan from the Hawaiian Ti plant. This polysaccharide was shown to possess, on the average, four terminal β-D-fructofuranosyl groups for every 14 hexose residues[23]. Agar-gel diffusion studies on the interaction of concanavalin A with this D-gluco-D-fructan, as well as with the several other levans investigated in the present work, have been published[4].

Studies in our laboratory have indicated that the combining sites of concanavalin A are complementary to the α-D-mannopyranosyl moiety, there being definite loci on the protein molecule for the hydroxyl groups on C-2, C-3, C-4, and C-6 of the pyranose ring and the α-D-glycosidic oxygen atom[24]. The α-D-glucopyranosyl moiety differs from the α-D-mannopyranosyl residue with respect only to the disposition of the 2-hydroxyl group, which, in the former species, is a destabilizing factor. For this reason, it is not surprising that 1,5-anhydro-D-mannitol (which contains four binding loci) is a good inhibitor of concanavalin A–polysaccharide interaction, being superior to 1,5-anhydro-D-glucitol (which contains only three binding loci).

For the purposes of the subsequent discussion, a generalisation may now be formulated: the minimum structural feature required for the binding of furanose sugars or their derivatives to concanavalin A is the 1,4-anhydro-D-arabinitol moiety. Thus, of the several 1,4-anhydropentitols and 2,5-anhydrohexitols (see Table II) that were examined as inhibitors of the concanavalin A–polysaccharide system, only 1,4-anhydro-D-arabinitol, 2,5-anhydro-D-glucitol, and 2,5-anhydro-D-mannitol were active. Similarly, only the methyl α- and β-D-arabinofuranosides (51 and 60 μmoles, respectively, for 50% inhibition), out of all the methyl pentofuranosides tested (see Table III), were inhibitors, these D-arabinofuranosides being superior to 1,4-anhydro-D-arabinitol (100 μmoles for 44% inhibition) and D-arabinose (110 μmoles for 20% inhibition). The enantiomorphic L-arabinose was, as expected, a noninhibitor.

Of the four methyl glycosides of D-fructose, three were found to be significant inhibitors of concanavalin A–polysaccharide interaction. Methyl β-D-fructopyranoside was the most potent inhibitor of precipitation (0.85 μmole for 50% inhibition), the inhibition being close to that of methyl α-D-mannopyranoside (0.60 μmole for 50% inhibition). Examination of the structure of this D-fructoside (see Fig. 6) reveals that it is configurationally related to 1,5-anhydro-D-mannitol. Methyl α-L-sorbopyranoside, which is configurationally similar to 1,5-anhydro-D-glucitol, is a poorer inhibitor than methyl β-D-fructopyranoside. Methyl α-D-fructopyranoside is essentially ineffective as an inhibitor.

The binding of furanoid sugars to concanavalin A may be rationalized on the basis of common configurational features with sugars possessing the pyranoid ring.

Thus, the disposition of the hydroxyl groups on C-3, C-4, and C-6 of the D-fructofuranosyl residue is similar to the orientation of the hydroxyl groups on C-3, C-4, and C-6 of the D-glucopyranosyl (or D-mannopyranosyl) residue. The greater potency of methyl β-D-fructofuranoside (16 μmoles for 50% inhibition) and 2,5-anhydro-D-mannitol (19 μmoles for 50% inhibition) over that of 2,5-anhydro-D-glucitol

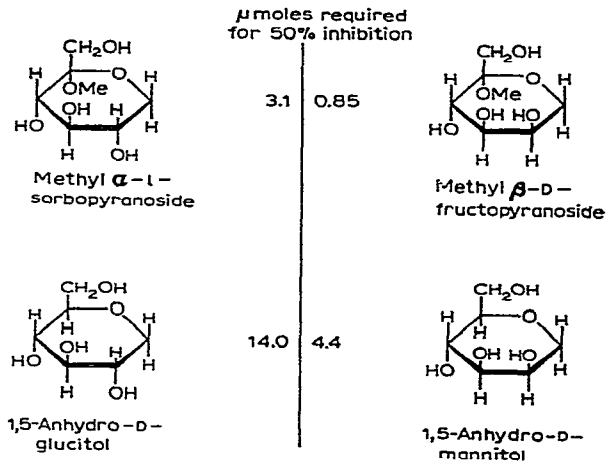


Fig. 6. Structural correlation and inhibition potency of two methyl hexulosides and two anhydroalditols.

(70 μmoles for 50% inhibition) suggests a further possibility, namely, binding at the 1-, 3-, and 4-hydroxyl groups of methyl β-D-fructofuranoside and 2,5-anhydro-D-mannitol. The 3-fold increase in inhibiting power of methyl β-D-fructofuranoside relative to that of 2,5-anhydro-D-mannitol, and the 4-fold increase in inhibiting power of methyl α-D-fructofuranoside relative to that of 2,5-anhydro-D-glucitol, indicate that the aglycon of these methyl D-fructofuranosides may in some way contribute to the binding; this, however, requires further investigation.

The binding of D-arabinose, D-fructose, and L-sorbose to the active sites of concanavalin A may be explained by the presence of the appropriate ring forms of these sugars in aqueous solution[25].

Inspection of Dreiding models of the D-glucopyranosyl and D-fructofuranosyl ring-systems reveals remarkable similarities in the angular dispositions of the critical hydroxyl groups of the two ring-systems (C. T. Bishop, personal communication). For example, if the hydroxyl group of the hydroxymethyl group is placed at its maximum distance from the neighboring, secondary hydroxyl group, with the line of vision down the C-5–H and C-3–H bonds, it is found that the angle subtended by O-6–O-4–O-3 in the D-glucopyranose ring is 121°. With the furanose ring in the T_2^3 conformation[26], the O-5–O-3–O-2 angle is exactly the same.

It is noteworthy that the β-D-fructofuranosyl ring, the residue present at the chain ends of levans, possesses only *three* definitive binding-sites with respect to the combining sites of the concanavalin A molecule, namely, the hydroxyl groups at

C-3, C-4, and C-6 (or C-1, C-3, and C-4). This situation contrasts with the *five* sites of attachment identified for the α-D-mannopyranosyl residue and the *four* sites identified for the α-D-glucopyranosyl moiety. Thus, it is not surprising that the interaction of levans with concanavalin A is of a lower order than that for α-D-mannans and α-D-glucans. It is, of course, essential that the frequency of branching and the number of glycosyl residues in the outer chains of a polysaccharide molecule be also considered in assessing the reactivity of carbohydrate polymers with concanavalin A.

Additional evidence showing that binding to the D-fructofuranose ring can occur at the 3-, 4-, and 6-hydroxyl groups is forthcoming from a consideration of the inhibition of precipitation observed with di-D-fructose 1,2′:2,1′-dianhydride (50% at 13.5 μmoles). This compound contains two D-fructofuranose ring systems[23,27,28] joined by 1,2′:2,1′ anhydride linkages; thus, the only possibility by which binding to concanavalin A can occur is at the hydroxyl groups at C-3, C-4, and C-6, or at C-3′, C-4′, and C-6′ of the sugar, or both. Since the hydroxyl groups at C-1 and C-2 of both of the D-fructofuranosyl residues are involved in anhydride ring-formation, simultaneous binding at positions C-1, C-3, and C-4 cannot occur.

Inhibition obtained with planteobiose indicates that binding of the D-fructofuranosyl ring to concanavalin A can also occur at the 1-, 3-, and 4-hydroxyl groups. Since D-galactose (8% inhibition at 104 μmoles) and melibiose [α-D-Gal*p*-(1→6)-D-G*p* (4% inhibition at 70 μmoles] were without effect on the precipitation, it follows that any inhibition observed with planteobiose must result from combination with the D-fructofuranosyl residue. In a solution of planteobiose, both the α- and β-D-furanose forms are likely to be present. Of these, only the β-D anomer would be expected to exhibit affinity for the receptor sites of concanavalin A. The low inhibition observed with planteobiose (25% at 35 μmoles) suggests that only a small proportion of the β-D anomer exists in an aqueous solution of this disaccharide. Quite interestingly, the trisaccharide planteose [α-D-Gal*p*-(1→6)-β-D-Fru*f*-(2↔1)-α-D-G*p*] does not produce any significant inhibition of precipitation at 35 μmoles, although the 3-, 4-, and 6-hydroxyl groups of the terminal α-D-glucopyranosyl residue, as judged by the Haworth structure, are free to interact. Inspection of a molecular model of this compound, however, suggested that the D-galactose residue may sterically block the essential hydroxyl groups on the nonreducing α-D-glucopyranosyl group.

A particularly interesting inhibitor of concanavalin A-polysaccharide precipitation is the disaccharide sucrose. In this sugar, both the α-D-glucopyranosyl group and β-D-fructofuranosyl group possess the necessary determinants for binding to the receptor sites of concanavalin A. Were both glycosyl groups actually involved in binding to the protein, sucrose would be expected to be a very potent inhibitor, assuming that both groups could occupy the active sites of concanavalin A without significant steric hindrance by the other. However, the affinity of sucrose for concanavalin A is of only the same order of magnitude as that of D-glucose (21 μmoles for 50% inhibition). Inhibition data obtained with "allosucrose" (52 μmoles for 0% inhibition), in which the D-glucosyl group of sucrose has been transformed into

a D-allosyl group, showed a greatly diminished inhibition, demonstrating that it is principally the α-D-glucopyranosyl group of sucrose that binds to concanavalin A.

Inhibition of concanavalin A–polysaccharide interaction by additional oligosaccharides containing D-fructofuranosyl residues indicated that the binding of sugars to concanavalin A may increase as additional D-fructofuranosyl groups are glycosidically linked to sucrose. Thus, inhibition of precipitation increases in the series of sucrose, 1-kestose, and nystose. Apparently, the serial addition of D-fructofuranosyl groups increases the number of potential binding-sites. As the series is ascended, the limiting factor may, perhaps, be the stabilization of the linear molecule by intramolecular hydrogen-bonds. It is still unknown whether a limit in inhibiting power may be attained. This is precisely the phenomenon that was observed when a homologous series of α-(1→2)-linked D-mannopyranosyl residues was examined as inhibitors of the concanavalin A–polysaccharide system[19].

It is interesting that inulin, which consists of a linear chain of about 35 (2→1)-linked β-D-fructofuranosyl residues terminated by a sucrose moiety, is not a precipitant of concanavalin A, but is an inhibitor of precipitation. The inhibiting power of inulin is of similar magnitude to that of methyl α-D-glucopyranoside, which is 2.5 times that of nystose.

Of the methyl ethers of D-fructose examined, only 3-*O*-methyl- and 4-*O*-methyl-D-fructose inhibited the concanavalin-A reaction significantly. Inhibition studies must be conducted on all of the possible methyl ethers of D-fructose before any further conclusions can be drawn.

The hapten-inhibition experiments described were conducted with the concanavalin A–dextran system. When these studies were repeated with levan as the precipitating polysaccharide (see Table I), the relative order of activity of the inhibitors tested was found to be precisely the same as that for the concanavalin A–dextran system, except that relatively smaller proportions of the individual saccharides were required. This result suggests that the binding of levan to concanavalin A is substantially weaker than that of dextran, and, hence, more readily inhibited. In both systems, methyl β-D-fructofuranoside (corresponding to the sugar residue at the chain end of levan molecules) is a poorer inhibitor than methyl α-D-glucopyranoside (corresponding to the sugar residue at the chain ends of α-D-glucans). The results of similar studies conducted with the concanavalin A–glycogen[9] and concanavalin A–D-mannan[19] systems parallel the inhibition pattern described for the concanavalin A–dextran system. These data provide strong evidence that the interaction of concanavalin A with polysaccharides occurs at the same receptor sites on the protein molecule. The fact that concanavalin A that had been isolated on Sephadex can be specifically bound to cross-linked, levan gel, and then subsequently be eluted with the same sugars employed to elute the protein from Sephadex supports this view.

ACKNOWLEDGMENTS

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PHYSICAL STUDIES ON OLIGOSACCHARIDES RELATED TO SUCROSE.

PART I. N.M.R. STUDIES ON THE PERACETATES OF SUCROSE, 1-KESTOSE, AND NYSTOSE*

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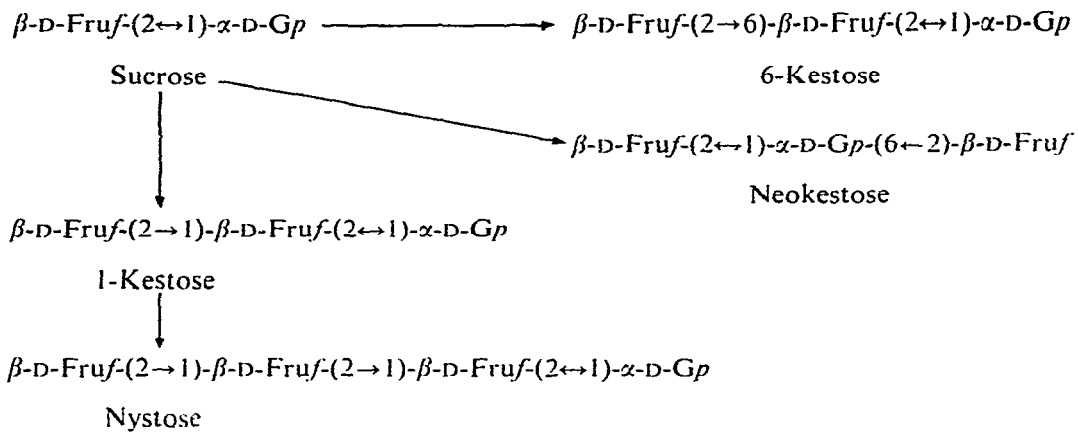
ABSTRACT

The n.m.r. spectra of sucrose octaacetate (**1**), 1-kestose hendecaacetate (**2**), and nystose tetradecaacetate (**3**) were measured in benzene-d_6, chloroform-*d*, and acetone-d_6, at 100 and 220 MHz. Signals of H-1, 2, 3, and 4 of the α-D-glucopyranosyl residue, and of H-3 and H-4 of the β-D-fructofuranosyl residue(s), could be observed individually for each of the compounds. The data confirm that all D-fructose residues in 1-kestose and nystose are in the furanoid ring-form. Observation of a two-proton, AB system near τ 6.3 (chloroform-*d*) in the spectrum of **2** provides diagnostic evidence for the (2→1) linkage between the two D-fructofuranosyl residues in 1-kestose [β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*]; other conceivable linkages are excluded. A second such AB system, observed in the spectrum of **3**, establishes that nystose is built up from 1-kestose by (2→1) attachment of an additional D-fructofuranosyl group. Other points of attachment are excluded, and the structure β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-G*p*, previously suggested for nystose, is confirmed. The n.m.r. signals for H-2, 3, and 4 of the α-D-glucopyranosyl residue in **1**, **2**, and **3**, and also in methyl α-D-glucopyranoside tetraacetate (**4**), showed close correlation.

INTRODUCTION

Sucrose can be regarded as the biogenetic precursor of a family of oligosaccharides found in sugar-cane and sugar-beet products. A molecule of sucrose acts as the acceptor, and sucrose provides the source of D-fructofuranosyl groups, for the enzyme-catalyzed generation of homologous D-fructofuranosyl derivatives of sucrose, as shown in Scheme I. Transfer of a β-D-fructofuranosyl group to O-6 of the D-fructosyl moiety of sucrose gives the trisaccharide 6-kestose[1,2], and transfer to O-6 of the

*Preliminary report, *Abstracts Papers Amer. Chem. Soc. Meeting*, 155 (1968) C23.



Scheme I

D-glucosyl moiety gives neokestose[2]. Transfer of the β-D-fructofuranosyl group to O-1 of the D-fructosyl moiety of sucrose gives the trisaccharide 1-kestose[2,3], and repetition of this process on the new terminal D-fructofuranosyl group gives the tetrasaccharide nystose[4]. The polysaccharide inulin can be regarded as resulting from an extension of the process exemplified by sucrose→1-kestose→nystose.

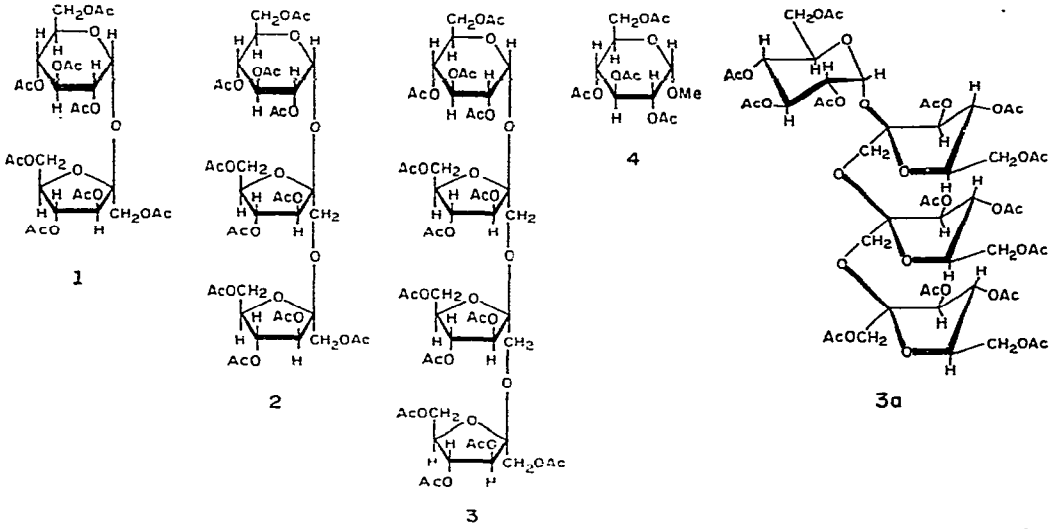
The linkage sequence in 1-kestose (see Scheme I) has been established by methylation and by partial hydrolysis[3], and the configurations of the linkages have been assigned on the basis of enzymic[3] and optical rotatory[4] evidence. Nystose, one of the products of the action of a fungal *alpha*-amylase on sucrose, has been shown[4] by fragmentation analysis and periodate oxidation to be an *O*-D-fructofuranosyl derivative of 1-kestose; the β-D linkage is indicated by optical rotatory data. By consideration of the normal specificity of the transglycosylase that produces nystose, the new β-D-fructofuranosyl group was presumed to be linked through O-1 of the terminal D-fructofuranosyl group of 1-kestose, to give the structure shown in Scheme I for nystose, but the position of this linkage has not been verified by a direct procedure.

As part of a broader program on the characterization, by modern physical methods, of oligosaccharides related to sucrose, the present article records a detailed study by n.m.r. spectroscopy at 100 and 220 MHz of the peracetates of sucrose, 1-kestose, and nystose. The data provide direct confirmation of the linkage positions in the structure already proposed for nystose.

DISCUSSION

The n.m.r. spectra of acetylated sugars can, in general, be subdivided[5] into three nonoverlapping regions, one of which is the acetyl-proton region near τ 8.0. The remaining protons, attached to the carbon atoms of the sugar residues, give rise to two discrete groups of signals. Signals appearing at fields higher than τ 5.5 arise from methylene groups and from protons of C_2CHOR (R ≠ Ac) groups (such as H-5 of hexopyranoses and hexulofuranoses), whereas resonances below τ 5.4 may

be assigned to the remaining methine protons (of C_2CHOAc groups, and anomeric protons, O_2CHR). On this basis, broad assignments can be made in regard to the n.m.r. spectra of sucrose octaacetate (**1**), 1-kestose hendecaacetate (**2**), and nystose tetradecaacetate (**3**). In the spectra of **1**, **2**, and **3**, at 100 MHz in chloroform-*d*



(see Fig. 1), the integrated intensities of the lowest-field (methine) groups of signals are 6, 8, and 10 protons, respectively. In each case, this multiplet includes the signals of H-1, H-2, H-3, and H-4 of the D-glucosyl group, together with the signals* of H-3′ and H-4′ of each D-fructose residue present. In the case of methyl α-D-glucopyranoside tetraacetate (**4**), a model compound for the acetylated α-D-glucopyranosyl group in **1**, **2**, and **3**, the H-1, 2, 3, and 4 signals likewise fall in this lowest-field region[6]. The signals for acetyl groups are observed as groups of closely spaced, three-proton singlets (some overlapping), having the anticipated intensities of 24, 33, 42, and 12 protons, for **1**, **2**, **3**, and **4**, respectively.

In the intermediate-field region (above τ 5.5, but below the acetyl-proton region), compound **4** shows signals of three protons (H-5 and two H-6 protons). Sucrose octaacetate (**1**) shows signals of 8 protons, because of 5 additional protons (H-1′$_s$ and H-6′$_s$ methylene protons, and H-5′$_s$) of the D-fructofuranosyl group. Addition of a second D-fructofuranosyl group introduces a further 5 protons resonating in this region, to give a total integral of 13 protons observed with 1-kestose hendecaacetate (**2**). Similarly, the addition of a third D-fructofuranosyl group introduces an additional

*Unprimed numbers refer to protons on the D-glucose moiety, and primed numbers refer to protons on an unspecified D-fructose residue. Protons on the same D-fructose residue are identified by the same letter (H-3a′, H-4a′, for example), except when the individual D-fructose residue is specifically identified. Protons on the D-fructofuranosyl group of sucrose are identified by a subscript "s", protons on the terminal D-fructofuranosyl group of 1-kestose are identified by a subscript "k", and protons on the terminal D-fructofuranosyl group of nystose are identified by the subscript "n".

5 protons, to give a total of 18 protons in the intermediate-field region for nystose tetradecaacetate (3).

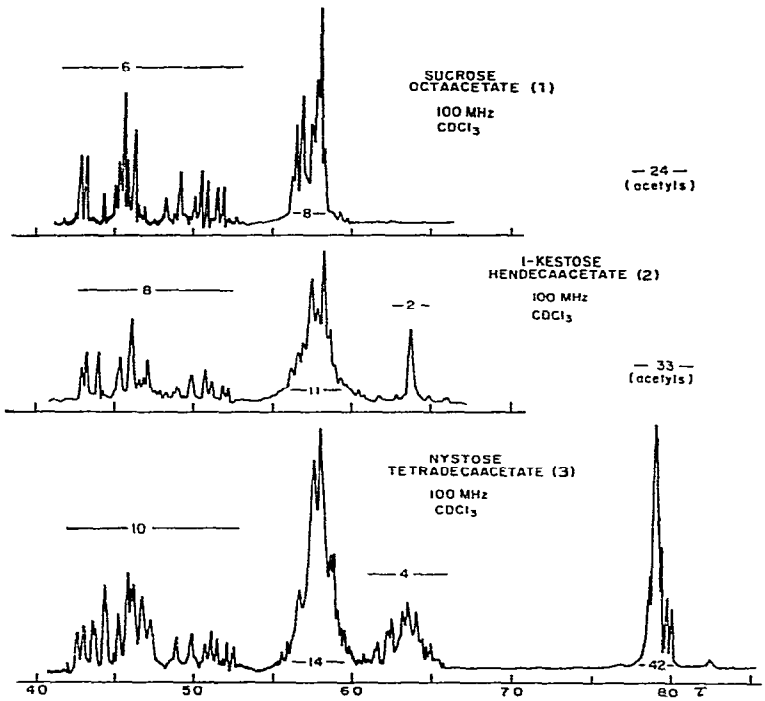


Fig. 1. N.m.r. spectra, at 100 MHz in chloroform-*d*, of sucrose octaacetate (1), 1-kestose hendecaacetate (2), and nystose tetradecaacetate (3); the acetyl-proton region of the spectra of 1 and 2 is omitted. Numbers on the curves denote integrated number of protons.

It is noteworthy that 2 and 3 contain methylene groups of two types, the C–CH_2OAc groups (also present in 1 and 4), together with methylene groups of the type C–CH_2OR (R ≠ Ac) that form bridges between the furanose rings. One such bridge is present in 2, and two are present in 3. The sugar residue (R) is not so strongly deshielding as is an acetyl group; in consequence, the signals of the "methylene bridges" can be observed in the τ 5.9–6.4 region, independent of other signals in the "intermediate" region. In the spectrum of 2 (see Fig. 1), the "intermediate" region shows an 11-proton multiplet together with a 2-proton, apparent singlet for the methylene bridge between the D-fructofuranosyl residues; in the spectrum of 3, the corresponding signals are a 14-proton multiplet together with a 4-proton multiplet for the two methylene bridges.

Detailed analyses of the low-field group of signals have been made by means of multiple-solvent techniques, nuclear-magnetic double-resonance, and the use of spectra measured at very high field-strength (equivalent to 220 MHz for protons). Assignments are presented in Tables I, II, and III, and are discussed in the following sections. The signals of the methylene bridges provide evidence for the linkage-

sequence in **2** and **3**. Other signals in the "intermediate" group are not readily analyzed.

Analysis of the lowest-field group of signals. — A. Sucrose octaacetate (**1**). The 100-MHz spectrum in chloroform-*d* (see Fig. 1) shows a quartet at τ 5.13, having spacings of 3.7 and 9.9 Hz, at the high-field end of this group of signals. These spacings, characteristic[6] of an axial proton coupled to a vicinal, axial proton on one side and a vicinal, equatorial proton on the other, identify the signal as H-2. From the spacings of the H-2 signal, the H-1 signal can be identified as a doublet at lowest field (τ 4.32), and the H-3 and H-4 signals can be identified as wide triplets; these assignments are readily confirmed by irradiating the H-2 signal and observing perturbation of the H-1 and H-3 signals. The remaining signals, a doublet at τ 4.55 and a triplet at τ 4.64, could be assigned to H-$3'_s$ and H-$4'_s$ on the basis of their signal multiplicities. These assignments are in agreement with those of Lemieux and Nagarajan[7] for **1**. It may be noted (see Table IV) that the chemical shifts of H-2, 3, and 4 are closely similar to those[6] of methyl α-D-glucopyranoside tetraacetate (**4**); the shifts of the H-1 signals for **1** and **4** differ, presumably because of the difference in the nature of the aglycon.

Much better separation of signals in this region could be achieved by use of benzene-d_6 as the solvent, and further resolution was obtained by measuring the spectrum at 220 MHz (see Fig. 2). Complete separation of the H-2, H-4, H-$3'_s$, and H-$4'_s$ signals was obtained, and the only overlap was between the H-1 and H-3 signals; as there is no appreciable spin-coupling between these protons, the first-order analysis is applicable. The first-order couplings (see Table II) can be expected to be very close to the absolute values. Relative to the signal positions in chloroform-*d*, the signals appear 0.2–0.4 p.p.m. to lower field in benzene-d_6, but the relative positions of the signals in the field are not changed. The signal positions with acetone-d_6 as the solvent were little different from those observed with chloroform-*d*, but the spectrum was not so well resolved.

B. 1-Kestose hendecaacetate (**2**). The low-field region of the spectrum of **2** in benzene-d_6, measured at 100 and 220 MHz (see Fig. 3), can be analyzed on a first-order basis; similar analyses can be made for the spectra measured in chloroform-*d* and acetone-d_6 (see Table I). The H-2 quartet is readily recognized; and the H-3 and H-4 triplets, and the H-1 doublet, can be identified by their spacings and by spin decoupling. The chemical shifts of H-2, 3, and 4 in chloroform-*d*, and also in benzene-d_6, correspond very closely (see Table IV) for the series methyl α-D-glucopyranoside tetraacetate (**4**), sucrose octaacetate (**1**), and 1-kestose hendecaacetate (**2**).

The remaining signals in the low-field group for **2** can be observed (benzene-d_6 at 220 MHz, Fig. 3) as an isolated doublet at τ 3.95, an isolated triplet at τ 4.38, and a doublet at τ 4.22 very close to the H-3 signal, with a triplet (τ 4.26) overlapping the doublet at τ 4.22. The doublets can be assigned to H-3′ signals, and the triplets to H-4′ signals. The doublet at τ 4.22 and the triplet at τ 4.26 arise from protons on different D-fructofuranose residues, because second-order effects between the two signals are not observed. The assignment of the doublet at τ 4.22 and the triplet at

TABLE I

CHEMICAL SHIFTS OF METHINE AND METHYLENE PROTONS OF PERACETYLATED SUCROSE, 1-KESTOSE, AND NYSTOSE[a]

Compound	*Solvent*	*Chemical shifts, τ*												
		D-*Glucose residue*				D-*Fructose residues*						*Methylene bridges*		*Other protons*
		H-1	*H-2*	*H-3*	*H-4*	*H-3a'*	*H-4a'*	*H-3b'*	*H-4b'*	*H-3c'*	*H-4c'*	*H-1a'*	*H-1b'*	*(integral)*
Sucrose octaacetate (1)	C_6D_6	4.15	4.99	4.22	4.71	4.33[b]	4.48[c]							5.43–5.89 (8)
	$CDCl_3$	4.32	5.13	4.55	4.93	4.55[b]	4.64[c]							5.62–5.95 (8)
	$(CD_3)_2CO$	4.25	5.12	4.48	4.91	4.44[b]	4.55[c]							5.53–5.95 (8)
1-Kestose hendecaacetate (2)	C_6D_6	3.92	4.87	4.15	4.67	4.22	4.38	3.95	4.26			6.01, 6.01		5.30–5.90 (11)
	$CDCl_3$	4.28	5.12	4.58	4.96	4.54	4.68	4.32	4.58			6.29, 6.38		5.58–5.93 (11)
	$(CD_3)_2CO$	4.19	5.10	4.50	4.92	4.47	4.59	4.18	4.49			6.12, 6.28		5.48–5.92 (11)
Nystose tetradecaacetate (3)	C_6D_6	3.87	4.90	4.17	4.65	4.19	4.37	3.98	4.30[d]	3.96	4.27[d]	5.81, 5.99	5.84, 6.00	5.35–5.78 (14)
	$CDCl_3$	4.28	5.17	4.60	4.97	4.55	4.68	4.42	4.67[d]	4.41	4.64[d]	6.22, 6.40	6.24, 6.42	5.54–5.97 (14)
	$(CD_3)_2CO$	4.19	5.11	4.50	4.91	4.48	4.59	4.26	4.54[d]	4.26	4.53[d]	6.05, 6.23	6.08, 6.27	5.51–5.86 (14)

[a]Data taken from 100- and 220-MHz spectra. [b]Identified as H-$3_g'$. [c]Identified as H-$4_g'$. [d]Not specifically differentiated.

TABLE II

FIRST-ORDER COUPLING-CONSTANTS FOR PERACETYLATED SUCROSE, 1-KESTOSE, AND NYSTOSE[a]

Compound	*Solvent*	*Coupling constants[b], Hz*										
		D-*Glucose residue*				D-*Fructose residues*						*Methylene bridges,*
		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{3a',4a'}$	$J_{4a',5a'}$	$J_{3b',4b'}$	$J_{4b',5b'}$	$J_{3c',4c'}$	$J_{4c',5c'}$	$J_{geminal}$
Sucrose octaacetate (1)	C_6D_6	3.7	10.5	9.5	9.7	5.5	5.1					
	$CDCl_3$	3.7	9.9	10.0	9.3	5.5	6.0					
	$(CD_3)_2CO$	3.9	10.3	10.6	9.3	5.5	5.0					
1-Kestose hendecaacetate (2)	C_6D_6	3.9	10.3	9.8	10.0	6.5	6.3	8.5	8.1			
	$CDCl_3$	3.9	9.0	9.4	10.0	7.0	6.0	8.0	8.0			9.5
	$(CD_3)_2CO$	3.7	10.5	10.1	9.8	6.0	6.2	7.8	6.9			9.5
Nystose tetradecaacetate (3)	C_6D_6	4.0	10.0	10.0	10.0	6.5	6.2	7.2	~7	7.2	~7	9.8, 9.6
	$CDCl_3$	3.5	10.0	9.5	9.8	6.8	~7	7.8	~7	7.8	~7	9.8, 9.5
	$(CD_3)_2CO$	4.0	10.6	9.5	10.0	6.4	~6.5	7.5	~7	7.5	~7	9.9, 9.4

[a]Data taken from 100- and 220-MHz spectra. [b]Values are considered accurate to ±0.5 Hz or better.

TABLE III

CHEMICAL SHIFTS OF ACETATE RESONANCES OF PERACETYLATED SUCROSE, 1-KESTOSE, AND NYSTOSE[a]

Compound	*Solvent*	*Chemical shifts of peaks observed, τ (integral)*
Sucrose octaacetate (1)	C_6D_6	8.07(3), 8.10(6), 8.17(3), 8.20(3), 8.25(3), 8.27(3), 8.32(3)
	$CDCl_3$	7.82(3), 7.88, 7.90(15), 7.95(3), 7.98(3)
	$(CD_3)_2CO$	7.86(3), 7.95(12), 7.98(3), 8.01(3), 8.04(3)
1-Kestose hendecaacetate (2)	C_6D_6	8.01(3), 8.02(3), 8.07(3), 8.10(3), 8.11(3), 8.13(3), 8.16(3), 8.25(3), 8.26(3), 8.33(3), 8.36(3)[b]
	$CDCl_3$	7.88(3), 7.91, 7.92(6), 7.93(12), 7.98(6), 8.01(3), 8.03(3)
	$(CD_3)_2CO$	7.87(3), 7.90(3), 7.93, 7.94(9), 7.96, 7.97(12), 8.02(3), 8.05(3)
Nystose tetradecaacetate (3)	C_6D_6	7.92(3), 7.99(3), 8.00(3), 8.01(3), 8.04(3), 8.12(9), 8.16(3), 8.27(3), 8.28(3), 8.32(3), 8.38(3), 8.39(3)[b]
	$CDCl_3$	7.87(6), 7.91(12), 7.92(12), 7.93(6), 7.95(6), 7.98(3), 8.02(3)
	$(CD_3)_2CO$	7.88(3), 7.90(9), 7.92(3), 7.95(3), 7.97(12), 8.00(3), 8.01(3), 8.03(3), 8.05(3)

[a]From 100-MHz spectra, unless otherwise stated. [b]Clear resolution of signals separated by 0.01 p.p.m. observed in 220-MHz spectra.

TABLE IV

COMPARATIVE, CHEMICAL-SHIFT DATA FOR THE D-GLUCOPYRANOSYL GROUP IN THE PERACETATES OF METHYL α-D-GLUCOPYRANOSIDE, SUCROSE, 1-KESTOSE, AND NYSTOSE

Compound No.	*Chemical shifts, τ*							
	Chloroform-d				*Benzene*-d_6			
	H-1	*H-2*	*H-3*	*H-4*	*H-1*	*H-2*	*H-3*	*H-4*
4[a]	(5.04)	5.11	4.51	4.93	(5.11)	4.96	4.20	4.73
1	4.32	5.13	4.55	4.93	4.15	4.99	4.22	4.71
2	4.28	5.12	4.58	4.96	3.92	4.87	4.15	4.67
3	4.28	5.17	4.60	4.97	3.87	4.90	4.17	4.65

[a]Data from ref. 6.

τ 4.38 to H-3a′ and H-4a′, respectively, of one D-fructofuranosyl residue, and the signals at τ 3.95 and 4.26 to H-3b′ and H-4b′ of the D-fructofuranosyl residue, is also supported by spin-coupling data (see Table II); the first pair (*a*) show $J_{3a',4a'} = 6.5$ Hz,

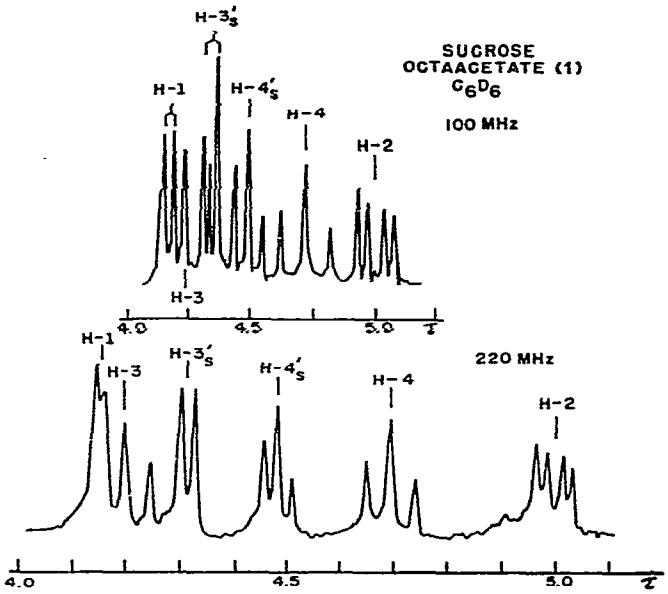


Fig. 2. The low-field portion of the n.m.r. spectrum of sucrose octaacetate (**1**) in benzene-d_6, at 100 and 220 MHz.

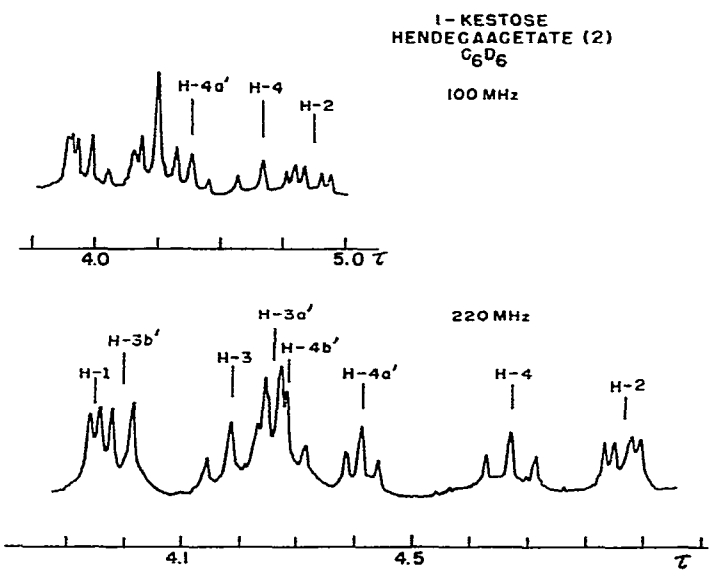


Fig. 3. The low-field portion of the n.m.r. spectrum of 1-kestose hendecaacetate (**2**) in benzene-d_6 at 100 and 220 MHz.

and the second pair (*b*) show $J_{3b',4b'} = 8.5$ Hz. The $J_{3s',4s'}$ coupling in sucrose octaacetate (**1**) in benzene-d_6 is 5.5 Hz. Similar groupings, in pairs, for H-3a′ and H-4a′, and H-3b′ and H-4b′, can be made from spectra measured in chloroform-*d* and acetone-d_6 (see Tables I and II), but the spectra are not so well resolved, and are readily analyzed only at 220 MHz.

It might be suggested that, by correlation with the chemical shifts and couplings observed with sucrose octaacetate (**1**) for H-3′$_s$ and H-4′$_s$, the H-3a′ and H-4a′ pair in **2** may be identified as H-3′$_s$ and H-4′$_s$, and the H-3b′ and H-4b′ pair as H-3′$_k$ and H-4′$_k$. However, it could equally well be argued that the shifts and couplings observed in **1** for H-3′$_s$ and H-4′$_s$, and in **2** for H-3a′ and H-4a′, are characteristic of a terminal D-fructofuranosyl group. Definitive assignment would require study of the spectrum of a sample of **2** that had been specifically deuterated[6,8] in one of the D-fructofuranosyl residues.

C. Nystose tetradecaacetate (**3**). The 100-MHz spectra were difficult to analyze completely in the low-field region, but the 230-MHz spectrum in benzene-d_6 (see Fig. 4), and the 220-MHz spectra in acetone-d_6 (see Fig. 5) or chloroform-*d*, showed the H-1, H-2, and H-4 signals separated from other signals, and the H-3 triplet could be extracted from a group of overlapping signals. The chemical shifts of H-1, 2, 3, and 4 in chloroform-*d*, benzene-d_6, and acetone-d_6 were almost identical with those of the corresponding signals observed for the lower homologs **2** and **1** (see Tables I and IV); the values for H-2, 3, and 4 were also very close to those observed with the monomer model **4** in the two solvents (chloroform-*d* and benzene-d_6) for which comparative data are available.

The H-3′ and H-4′ signals for the three D-fructofuranosyl residues in **3** were identified from the spectrum in benzene-d_6 (see Fig. 4) as doublets at τ 3.96, 3.98, and 4.19, and as triplets at τ 4.27, 4.30, and 4.37. From coupling data and the absence of second-order effects, the doublet at τ 4.19 and the triplet at τ 4.37 could be assigned to the same D-fructofuranosyl residue (H-3a′ and H-4a′). The doublets at τ 3.96 and 3.98 had almost identical splittings, so that correlation of each with the individual triplets at τ 4.27 and 4.30 (to differentiate the pairs H-3b′ and H-4b′, and H-3c′ and H-4c′) could not be made.

The pair of signals H-3a′ and H-4a′ have chemical shifts considerably different from those for the other two pairs (H-3b′ and H-4b′, H-3c′ and H-4c′), suggesting that H-3a′ and H-4a′ arise from the terminal D-fructofuranosyl group (H-3′$_n$ and H-4′$_n$), and the other two pairs arise from the inner D-fructofuranosyl residues (H-3′$_k$ and H-4′$_k$, H-3′$_s$ and H-4′$_s$), but this rationalization cannot be proved on the basis of the data at present available.

The H-3b′ and H-3c′ signals were observed, in chloroform-*d* at 220 MHz, as low-field doublets of almost identical chemical-shifts; in acetone-d_6 (see Fig. 5), the two signals coincided exactly. The signals of H-3a′, H-4a′, H-4b′, and H-4c′ were more difficult to identify in the spectra measured in chloroform-*d* or acetone-d_6 than in the spectra in benzene-d_6, because of more extensive overlap of signals in the first two solvents.

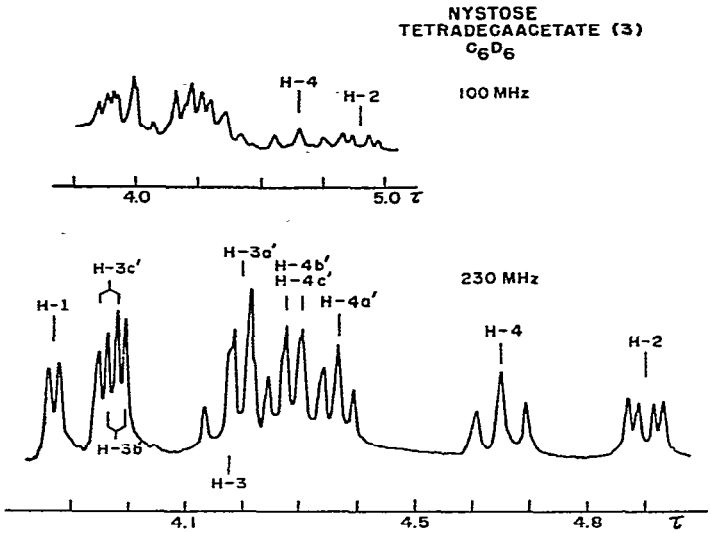


Fig. 4. The low-field portion of the n.m.r. spectrum of nystose tetradecaacetate (3) in benzene-d_6 at 100 and 230 MHz.

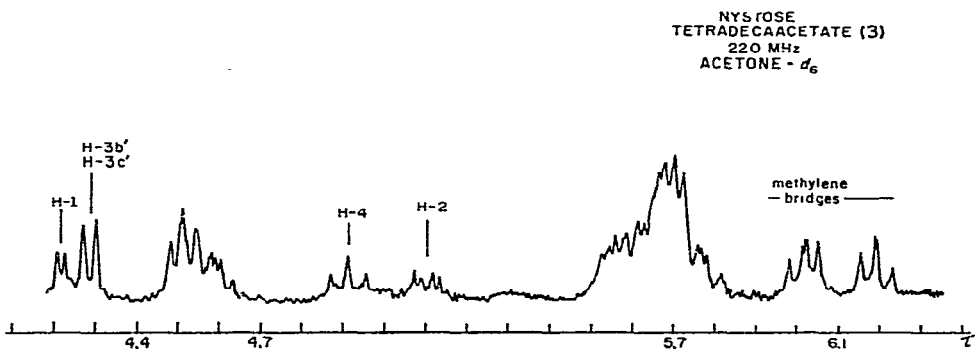


Fig. 5. The low-field portion of the 220 MHz spectrum of nystose tetradecaacetate (3) in acetone-d_6.

Coupling constants. — The coupling constants $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ observed for the D-glucopyranosyl group in **1**, **2**, and **3** (see Table II) are unexceptional; they closely resemble the couplings observed[6] for methyl α-D-glucopyranoside tetraacetate (**4**), and accord entirely with the anticipated *Cl*(D) conformation of the pyranoid ring in **1**, **2**, **3**, and **4**. Some variation in the values of $J_{3',4'}$ for the D-fructofuranose residues is noted with **1**, **2**, and **3**, probably representing minor deviations from a favored conformation[7] having C-2′, C-3′, C-5′, and O-5′ approximately in a plane, with C-4′ displaced above the plane (as viewed when the ring is numbered clockwise). Such a conformation (*a*) brings H-3′ and H-4′ into a quasi-axial arrangement, giving rise to large values of $J_{3',4'}$ and $J_{4',5'}$, (*b*) allows the substituents at C-3′, C-4′, and C-5′ to adopt quasi-equatorial orientations, and (*c*) allows O-2′ to

adopt a quasi-axial orientation for maximum relief from polar destabilization (anomeric effect) while allowing C-1′ to become quasi-equatorial. The coupling data do not establish the favored relative orientations of the different rings in **1**, **2**, or **3**, and no such conformational significance is implied in the representation **3a** for **3**.

Signals of acetyl-methyl protons. — Of the three solvents used in this study, benzene-d_6 gives the greatest spectral dispersion. All eleven acetyl-group signals are observed separately in the spectrum of **2** in benzene-d_6, even at 100 MHz (see Table III). In **1**, two of the eight acetyl-group signals overlapped in the 100-MHz spectrum, and in the spectrum of **3** there were three coincident resonances among the fourteen acetyl groups, even at 230 MHz. A greater degree of signal overlap was observed with spectra measured in chloroform-*d* and in acetone-d_6. In benzene-d_6, some of the acetyl-group signals were highly shielded, as observed with other acetylated sugars[6,8,9].

Although the acetyl-group assignments for the reference compound **4** have been established definitively by synthesis of specifically deuterated derivatives, no such firm assignments have been made for **1**, **2**, and **3**, and suggested assignments made on a comparative basis with data[6] for **4** may not be reliable. The excellent dispersion of the acetate spectral-region observed in the present work with **1**, **2**, and **3** suggests that firm assignment of acetyl-proton signals in **1**, as obtained by study of specifically deuterated derivatives, would provide a useful method for determining the relative reactivities of the different hydroxyl groups in sucrose by the general procedure described[6] for methyl α-D-glucopyranoside.

Signals of the methylene bridges in **2** *and* **3**. — The signals of the methylene-bridge protons in **2** and **3**, in benzene-d_6, chloroform-*d*, and acetone-d_6, are depicted in Fig. 6. Chemical-shift data are given in Table I, and coupling constants are given in Table II. In 1-kestose hendecaacetate (**2**), the two protons of the methylene bridge are slightly nonequivalent in chloroform-*d*, so that, at 220 MHz, an AB quartet

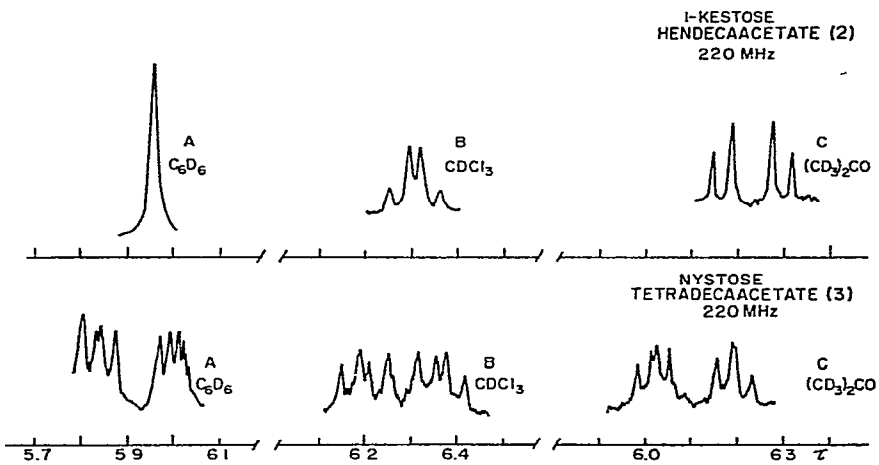


Fig. 6. Signals of the inter-residue methylene bridges of 1-kestose hendecaacetate (**2**) and nystose tetradecaacetate (**3**) in (*A*) benzene-d_6 at 230 MHz, (*B*), chloroform-*d* at 220 MHz, and (*C*) acetone-d_6 at 220 MHz.

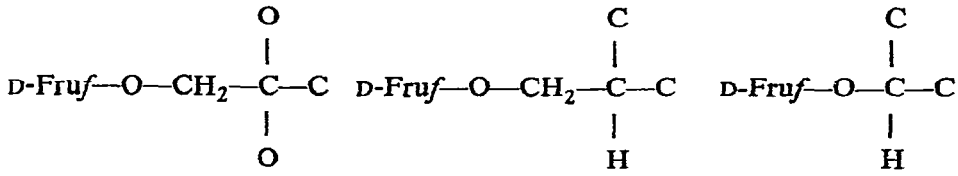
exhibiting a geminal coupling of 9.5 Hz is observed. The nonequivalence is more pronounced in acetone-d_6, but, in benzene-d_6, the protons are practically equivalent, so that an apparent singlet is observed. Nonequivalence of methylene protons is of common occurrence when the methylene group is attached to an asymmetrically substituted carbon atom[10].

Two methylene bridges are present in nystose tetradecaacetate (3), and, in each of the three solvents, the methylene protons in each bridge are nonequivalent. In chloroform-*d*, an 8-line pattern due to the four doublets of two different AB quartets is observed, and a similar pattern is noted in the spectrum measured in benzene-d_6. In acetone-d_6, a similar pattern also arises, but near-superposition of two peaks gives rise to an apparent triplet instead of a pair of doublets. The geminal couplings are, in each case, approximately 9.6 Hz, so that pairs of doublets cannot be correlated on the basis of couplings. However, the appearances of the signals indicate that geminal protons give one signal in the lower-field pair of doublets and one in the higher-field pair; a strongly perturbed pattern would have resulted had adjacent signals arisen from spin-coupled protons.

Structural significance of the n.m.r. data. — The foregoing analyses indicate how a combination of multiple-solvent techniques, measurements at high field-strengths, and the use of spin decoupling permits extensive and detailed interpretations of n.m.r. spectra, even for a compound such as 3, containing 70 protons. The recorded data may be of use in structural characterization of other oligosaccharides. The comparative study of the polymer-homologous series **1**, **2**, and **3** provides a basis for the interpretation of n.m.r. spectra of the acetates of inulin and bacterial levans.

There is little reasonable doubt that the linkage sequence accepted for 1-kestose[3] is correct. Nevertheless, given that 1-kestose is an *O*-D-fructofuranosylsucrose, the observation of the "methylene bridge" signals as an AB pattern establishes independently that the *O*-D-fructofuranosyl group is attached to O-1′ of sucrose. Had the *O*-D-fructofuranosyl group been attached at one of the secondary hydroxyl groups of sucrose, all four methylene groups would have been attached to an acetoxy group (C—CH_2OAc), and no signals in the "methylene bridge" region would have been observed (see Scheme II). Had the *O*-D-fructofuranosyl group been attached to O-6 or O-6′ of sucrose, the signals of a methylene bridge would have been observed because the 6 (or 6′) methylene group would be shielded at least as strongly as those of the 1′ methylene group in the accepted structure **2**. However, attachment through O-6 or O-6′ (see Scheme II) would give a methylene group having a vicinal proton (H-5 or H-5′), so that the signals of the methylene bridge would be observed as the AB part of an ABX system, and not as a simple AB pattern. The AB pattern observed in the "methylene bridge" region serves, therefore, to identify, uniquely, the linkage position of the terminal D-fructofuranosyl group in 1-kestose.

Nystose is an *O*-D-fructofuranosyl derivative of 1-kestose, and the chemical and enzymic evidence so far advanced does not prove the point of attachment of the terminal D-fructofuranosyl group; eleven different positions are available for the linkage, although the $1'_k$ position is considered the most probable point of attachment.



Linkage through O-1′ | Linkage through O-6 or O-6′ | Linkage through a secondary hydroxyl group

Scheme II

The fact that signals of *two* methylene bridges are observed in the spectrum of **3** establishes that the linkage is not through any one of the seven secondary hydroxyl groups of 1-kestose. Furthermore, the fact that the methylene-bridge signals are observed as a pair of AB patterns establishes that the terminal *O*-D-fructofuranosyl group is not attached to O-6, O-$6'_s$ or O-$6'_k$ of 1-kestose, because, in such a situation, one of the methylene bridges would have given a more complex pattern through coupling with H-5, H-$5'_s$, or H-$5'_k$. The pattern of signals observed for the methylene bridges in **3** provides direct verification, therefore, for the linkage pattern previously suggested[4] for nystose.

EXPERIMENTAL

Procedures. — Column chromatography was performed with an acetone-washed mixture of 5 parts (by wt.) of a hydrated magnesium acid silicate, Magnesol (manufactured by the Westvaco Chemical Division of the Food Machinery and Chemical Corp., South Charleston, West Virginia), with 1 part of Celite No. 535 (produced by the Johns-Manville Corp., New York, New York). The chromatograms were developed with 25:1 (v/v) benzene–2-methyl-2-propanol. Zones were detected with a 1% solution of potassium permanganate in 10% sodium hydroxide. Components were recovered from the adsorbent by use of acetone. T.l.c. was performed[11] with silica gel, ~100 μm thick, on polyester sheets (Eastman Kodak Co., Rochester, N. Y.), with 25:1 (v/v) benzene–isopropyl alcohol as the developer and 1-naphthol–phosphoric acid as the indicator. N.m.r. spectra were measured at room temperature with a Varian HA-100 n.m.r. spectrometer, with tetramethylsilane ($\tau = 10.00$) as the internal standard. N.m.r. spectra at 220 MHz were measured with a Varian HR-220 spectrometer equipped with a superconducting solenoid (compare F. A. Nelson and H. E. Weaver, "High Resolution Superconducting Spectrometer", presented at the International Conference on Magnetic Resonance and Relaxation, XIVth Colloque Ampère, Ljubljana, Yugoslavia, September 9th, 1966).

Materials. — *A. Sucrose octaacetate* (**1**). The commercial material (Matheson Coleman & Bell, Norwood, Ohio) was recrystallized twice from 95% ethanol; m.p. 85–86°, $[\alpha]_D^{20}$ +60° (*c* 4, chloroform) [lit.[12] m.p. 89°, $[\alpha]_D^{20}$ +59.6° (chloroform)].

B. 1-Kestose hendecaacetate (2). A mixture of twice recrystallized 1-kestose[3] (500 mg), powdered, fused sodium acetate (500 mg), and acetic anhydride (5 ml) was heated at 80–90° until dissolution was complete (~2 h), and for an additional 2 h. The solvent was removed under diminished pressure at the same temperature, chloroform (15 ml) was added to the residue, and the mixture was washed with saturated, aqueous sodium hydrogen carbonate at 10° until all of the acetic anhydride had been removed. The chloroform solution was dried (sodium sulfate), and evaporated to give **2** as a chromatographically homogeneous syrup; yield 850 mg 88.7%); $R_{sucrose\ octaacetate}$ 0.58–0.59. To ensure the highest degree of homogeneity, the product was purified by column chromatography[13], to give the pure acetate **2** as a colorless syrup, $[\alpha]_D^{20}$ +31.8 ±0.3° (*c* 3.7, chloroform).

Anal. Calc. for $C_{40}H_{54}O_{27}$: C, 49.69; H, 5.63. Found: C, 49.69; H, 5.90.

C. Nystose tetradecaacetate (3). Acetylation of five-times recrystallized nystose[4] (304 mg) by the procedure used for **2**, followed by column-chromatographic purification, gave the acetate **3** as a chromatographically homogeneous syrup, yield 500 mg (87.2%), $[\alpha]_D^{20}$ +19.1 ±0.3° (*c* 2.0, chloroform); $R_{sucrose\ octaacetate}$ 0.33–0.34.

Anal. Calc. for $C_{52}H_{70}O_{35}$: C, 49.76; H, 5.62. Found: C, 49.90; H, 5.89.

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THE ISOLATION OF L-IDURONIC ACID FROM THE CRYSTALLINE BARIUM ACID SALT OF HEPARIN[1]

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ABSTRACT

The thrice-recrystallized barium acid salt of heparin was hydrolyzed by a modified Monier-Williams method with 72% sulfuric acid at 25°, followed by dilution and refluxing. The uronic acid fraction (13%) of the hydrolyzate was chromatographically shown to contain D-glucuronic acid, L-iduronic acid, and their lactones. The L-iduronolactone component was isolated, and characterized as the crystalline brucinium salt of its acid. It was established that the L-iduronic acid was derived neither from contaminant dermatan sulfate nor through C-5 epimerization of D-glucuronic acid. Previous hydrolytic conditions (which gave, in addition to 2-amino-2-deoxy-D-glucose, only D-glucuronic acid or D-glucose from purified heparin or its partially carboxyl-reduced derivatives, respectively) are now considered to have been too drastic to give all of the representative, degradation fragments.

INTRODUCTION

Heparin is a heteropolymer containing uronic acid and 2-amino-2-deoxy-D-glucose residues as its components. However, many attempts to determine the identity of its uronic acid moieties have not yet afforded conclusive results. The small proportion (10%) of D-glucuronic acid obtained after partial hydrolysis with acid[2], and the low yield of D-glucose isolated from the hydrolyzates of several carboxyl-reduced heparin derivatives[3,4], have been regarded as support for earlier conclusions[5] that heparin contains only one uronic acid component. However, there is some evidence that another uronic acid is present, in addition to D-glucuronic acid. By means of paper chromatography, Cifonelli and Dorfman[6] have detected L-iduronic acid in the hydrolyzate of heparin purified by cetylpyridinium chloride. Other investigators have considered that L-iduronic acid is a structural unit of heparin, but have based their evidence largely on commercial preparations of equivocal purity[7-10]. In a continued effort in this laboratory to elucidate the fine structure of heparin definitively, L-iduronic acid has now been isolated, and characterized by mild, acid hydrolysis of the thrice-recrystallized barium acid salt of heparin.

RESULTS AND DISCUSSION

Commercial heparin, of good quality, was converted into the crystalline barium acid salt[11,12]; this was recrystallized three times, and was then reconverted

into its sodium salt. In this work, contact with hot acetic acid was minimized, and analyses showed no loss in sulfate content. The heparin so purified was hydrolyzed by a modification of the method of Monier-Williams[13] (for cellulose) by use of 72% sulfuric acid for 2 h at 25°, followed by dilution to an acid concentration of 3% and refluxing for 2.5 h. The acids in the hydrolyzate were neutralized with barium carbonate, the suspension was filtered, and the inorganic ions and the amino sugar components were removed from the filtrate by means of a cation-exchange resin. Concentration of the effluent from the resin column gave the syrupy uronic acid fraction (yield, 13% of theoretical). Paper chromatography showed that this syrup contained much L-iduronolactone, together with smaller proportions of L-iduronic acid, D-glucuronic acid, and D-glucuronolactone. The apparent preponderance of the total L-iduronic acid in the syrup was probably due to the greater susceptibility of the L-idosyluronic than the D-glucosyluronic linkage to hydrolysis with acid. The L-iduronolactone fraction was then isolated from the syrup by preparative paper-chromatography (yield 32% of the syrup, or 4.5% of the theoretical, based on heparin). Attempted crystallization of this lactone fraction was unsuccessful, and rechromatography showed that the free acid form was also present, as a result of rapid, lactone–acid equilibration. After a sample of this lactone–acid mixture had been reduced with sodium borohydride[14] in an acetate buffer, paper chromatography indicated that the product contained mainly L-idonic acid, L-idonolactone, and L-iditol, as well as a small proportion of L-idose. The syrupy equilibrium mixture (optical rotatory power observed) of L-iduronolactone and its free acid was then converted into a crystalline brucinium salt which exhibited an X-ray powder diffraction pattern identical with that of the brucinium salt of authentic L-iduronic acid.

That the L-iduronic acid isolated could not have originated from contaminant dermatan sulfate was demonstrated by Sephadex polymer assay[15], as well as by the absence of *N*-acetyl groups (by analysis[16], and in the n.m.r. spectra). This absence of acetyl verifies previous results[12,17]. Excellent separation was achieved when an artificial mixture of the purified heparin with dermatan sulfate was applied to a Sephadex column. Under identical conditions, the purified heparin, alone, gave only one distinct component. The absence of dermatan sulfate was also corroborated by control experiments involving the nitrous acid deamination reaction: applied to a polysaccharide containing 2-amino-2-deoxyhexose residues, this reaction would lead to the formation of a 2,5-anhydrohexose residue from the parent, amino sugar residue, with simultaneous scission of the adjacent glycosidic bond, resulting in fragmentation of the original polymer[18]. However, no deaminative scission would occur with such mucopolysaccharides as chondroitin sulfate or dermatan sulfate, whose amino groups are blocked by the acetyl function. De-*N*-sulfated heparin, prepared from the purified sample, was then treated with nitrous acid; essentially all of the product (99%) was found to be dialyzable. The validity of the deamination reaction was further tested with chondroitin 6-sulfate (instead of heparin); no dialyzable carbohydrate material was then detected, and 98% of the original polysaccharide was recovered.

A possible C-5 epimerization of the D-glucuronic acid residues in heparin should be considered, because Fischer and Schmidt[19] obtained L-iduronic acid by heating a neutral, aqueous solution of sodium D-glucuronate. Consequently, sodium chondroitin 6-sulfate and sodium D-glucuronate were separately treated by the modified, sulfuric acid method[13], but no trace of L-iduronic acid in the resulting solution was detected by paper chromatography. Similarly, no C-5 epimerization was observed when L-iduronic acid was treated in the same way. These results eliminated the possibility that the L-iduronic acid and D-glucuronic acid found in the heparin hydrolyzate obtained by the sulfuric acid method[13] were products of C-5 inversions under the experimental conditions used.

Although the absence of dermatan sulfate or of another polysaccharide containing an *N*-acetyl group had now been established, the possibility still remained that the L-iduronic acid was derived from an unknown polysaccharide hitherto not separated from the exhaustively purified heparin. However, moving-boundary electrophoresis of a heparin sample purified through the barium acid salt had indicated[20] only one component, and therefore, the existence therein of an unknown polysaccharide is highly improbable.

The L-iduronic acid residues are present in heparin in addition to the residues of D-glucuronic acid (previously found in the hydrolyzate). However, the L-iduronic acid had not been detected in previous work from this laboratory[3,21]. Consequently, this work[3] has been repeated, and the observations reported therein have been found to be reproducible. When the same carboxyl-reduced heparin was hydrolyzed by the modified, sulfuric acid method[13], L-idose and L-iduronic acid were readily detected among the other products anticipated. Therefore, the formula for heparin depicted in the previous publications[3,21] should be modified to accommodate the present finding. Apparently, the hydrolytic conditions employed in the previous work in this laboratory[3,5,21] and adopted by others[4,22] are too drastic to give all of the representative, acid-degradation fragments from heparin.

The evidence presented herein therefore establishes, on a crystalline, isolative basis, the presence of L-iduronic acid residues in the thrice-recrystallized barium acid salt of heparin.

EXPERIMENTAL

General methods. — Descending paper-chromatography was effected on Whatman No. 1 paper with 4:1:2 (v/v) butyl alcohol–acetic acid–water (solvent A), 5:5:3:1 (v/v) ethyl acetate–pyridine–water–acetic acid (solvent B), and 40:11:49 (v/v) butyl alcohol–ethanol–water (solvent C). Zones were located with silver nitrate–sodium hydroxide, naphthoresorcinol, or aniline hydrogen phthalate reagents. R_G denotes the rate of chromatographic movement relative to that of D-glucose. All evaporations were performed under diminished pressure below 45°. Analyses were made by W. N. Rond. N.m.r. spectra were recorded by J. B. Morton with a Varian A-60 n.m.r. spectrometer for solutions in deuterium oxide, with sodium

4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. Melting points were obtained with a Thomas–Hoover apparatus. Specific rotations were determined in a 1-dm polarimeter tube. Infrared spectra were recorded with a Perkin–Elmer Infracord infrared spectrometer. X-Ray powder diffraction data give interplanar spacings in Å for CuKα radiation, λ 1.539 Å, Ni filter, 114.6 mm camera diameter, with photographic recording. Relative intensities were estimated visually: m, medium; s, strong; v, very; w, weak. The strongest lines are numbered (1, strongest); multiple numbers indicate approximately equal intensities.

Purification of heparin. — The commercial heparin used in this investigation was kindly supplied by The Upjohn Company, Kalamazoo, Michigan (lot MG 233, sodium salt); 126 I.U./mg; $[\alpha]_D^{29}$ +44° (*c* 1.0, water).

Anal. Calc. for $C_{24}H_{31}N_2Na_7O_{35}S_5$: C, 23.45; H, 2.52; N, 2.28; S, 13.04. Found: C, 23.03, H, 2.86; N, 2.35; S, 12.62.

The further purification procedure was essentially the same as that previously reported[12], except that contact with warm acetic acid was minimized. The commercial heparin (9.0 g) was dissolved in water (360 ml), and a 5% aqueous barium acetate solution (120 ml) was added. The clear, slightly yellowish solution was heated to 65°, and 90 ml of acetic acid was added. On gradual cooling to room temperature, clusters of fine needles separated; these were collected by filtration. The first crop was dissolved in the minimal volume of water at 65°, and acetic acid was slowly added to incipient turbidity; crystals were obtained after the solution had been cooled to room temperature. This procedure was twice repeated, and the crop of crystals was washed successively with 80% acetic acid, absolute ethanol, and ether, and dried under diminished pressure to give 6.9 g of the thrice-recrystallized barium acid salt. This salt was then reconverted into the sodium salt by passage through a column (2.5 × 25 cm) of Dowex-50 (Na^+); the column was washed with water, and the eluate and washings were combined, dialyzed against running water for 3 days, and freeze-dried, to give 5.4 g (overall yield 60%) of purified sodium salt, $[\alpha]_D^{21}$ +48° (*c* 1.0, water).

Anal. Calc. for $C_{24}H_{31}N_2Na_7O_{35}S_5$: C, 23.45; H, 2.52; N, 2.28; S, 13.04; CH_3CO, 0.0. Found: C, 23.85; H, 2.33; N, 2.40; S, 12.65; CH_3CO (ref. 16), 0.0. The n.m.r. spectrum (D_2O) showed no signal for CH_3CO protons, whereas a 1:1 mixture of heparin and dermatan sulfate gave a broad singlet (τ 8.13).

Sephadex-gel fractionation. — Purified heparin (5 mg) was introduced onto a column (2 × 20 cm) of DEAE-Sephadex gel (Cl^-) (A-25, capacity 3.5±0.5 meq/g, particle size 40–120 μm), and the column was eluted with aqueous sodium chloride followed by sodium chloride in 0.01N hydrochloric acid, according to Schmidt[15]. Fractions (5 ml) were analyzed by the Dische carbazole method[23]. Heparin was eluted at fractions 26–35 as a single peak. As a reference experiment, a 1:1 mixture (10 mg) of heparin and dermatan sulfate was fractionated in the same way; dermatan sulfate and heparin were eluted at fractions 17–24 and 26–35, respectively.

Nitrous acid deamination of de-N-sulfated heparin. — Purified heparin (2.00 g) was heated in 0.04N hydrochloric acid (100 ml) for 2 h at 95–100°. The hydrolyzate was cooled, made neutral with sodium hydrogen carbonate, dialyzed against running

water for 2 days, and freeze-dried, to give 1.35 g of a creamy white powder. This de-*N*-sulfated heparin (1.00 g) was dissolved in 35 ml of 250 mM sulfuric acid, and a solution of 3.7 g of barium nitrite in 10 ml of water was gradually added, with occasional shaking. The mixture was kept for 48 h at room temperature, a slight excess of sulfuric acid was added (to remove barium ions), and the residual nitrous acid was removed by evaporation under diminished pressure. The excess of sulfuric acid was neutralized with barium carbonate, the precipitate was removed by centrifugation, and the supernatant liquor was dialyzed against running water for 3 days, and freeze-dried, to give 12.6 mg (0.6%) of nondialyzable material.

As a control experiment, 1.00 g of chondroitin 6-sulfate was treated in the same way with 0.04N hydrochloric acid and then with nitrous acid; evaporation of the dialyzable portion gave no carbohydrate material, and 98% of the polysaccharide was recovered.

Hydrolysis of purified heparin. — The purified heparin (4.50 g) was dissolved in 18 ml of 72% sulfuric acid[13] with vigorous stirring for 2 h at 25°. The viscous solution was diluted to an acid concentration of 3%, and boiled for 2.5 h under reflux. The hydrolyzate was cooled to room temperature and made neutral with barium carbonate, and the precipitate was removed by centrifugation. The yellowish, supernatant liquor was passed through a column (2.5 × 25 cm) of Dowex-50 (H^+), and the column was washed with water. The eluate and washings were combined, and evaporated to dryness, to give 180 mg (13%) of a light-brown syrup, which showed two major spots and two minor spots on paper chromatograms; R_G (solvent A) 0.70 (D-glucuronic acid), 1.21* (L-iduronic acid), 2.18 (D-glucuronolactone), 3.23* (L-iduronolactone); R_G (solvent B) 0.55 (D-glucuronic acid), 0.77* (L-iduronic acid), 1.62 (D-glucuronolactone), and 1.79* (L-iduronolactone); R_G (solvent C) 0.65 (D-glucuronic acid), 0.99* (L-iduronic acid), 1.77 (D-glucuronolactone), and 2.53* (L-iduronolactone).

As a control experiment, sodium D-glucuronate and sodium chondroitin 6-sulfate were treated by the modified, sulfuric acid method[13], and the hydrolyzates were processed in the same way as for purified heparin. Paper chromatography (solvent A) showed two spots, of R_G 0.70 (D-glucuronic acid) and R_G 2.18 (D-glucuronolactone). A solution obtained by removal of the isopropylidene group from 1,2-*O*-isopropylidene-L-idofuranurono-6,3-lactone[24,25] by hydrolysis with 1N hydrochloric acid for 2 h at 100° gave two spots, of R_G 1.21 (L-iduronic acid) and R_G 3.23 (L-iduronolactone) by the same treatment.

Isolation of L-iduronic acid as its crystalline brucinium salt. — The syrup (180 mg) obtained from the heparin hydrolyzate was applied to Whatman No. 3MM filter papers, and developed with solvent A. The zones corresponding to L-iduronolactone were excised, by use of guide strips, and extracted with the same solvent. The solution was decolorized, and evaporated to dryness, giving 58 mg (32% of the mixture, or 4.5% of the theoretical, based on heparin) of syrupy L-iduronolactone, $[\alpha]_D^{23}$ 0→+25°

*Represents a strong spot.

(3 days, *c* 1.0, water); lit.[25] for free acid, $[\alpha]_D^{22}$ +33° (4 h, *c* 3, water); ν_{max}^{Nujol} 2.8–3.1 (OH), 5.55 and 8.44 (lactone C=O), 7.97, 8.91, 9.4–9.7, 10.52, 11.09, 11.57, 12.22, 12.45, and 13.52 μm.

Anal. Calc. for $C_6H_8O_6$: C, 40.91; H, 4.58. Found: C, 41.30; H, 4.59.

On being chromatographed, an aqueous solution of the syrup showed two spots, corresponding to L-iduronic acid and L-iduronolactone, apparently due to lactone–acid equilibrium.

The syrupy L-iduronolactone (30 mg) was dissolved in 1 ml of acetate buffer (pH 6), and 50 mg of sodium borohydride[14] was slowly added, with stirring. After 15 min, the mixture was passed through a small column of Dowex-50 (H^+), and the column was washed with water. The eluate and washing were combined, and evaporated to dryness, and the residue was thrice dissolved in methanol and evaporated to dryness (to remove boric acid). The syrupy product was examined by paper chromatography (solvent A), R_G 0.73* (L-idonic acid), 1.03* (L-iditol), 1.58 (L-idose), and 2.03* (L-idonolactone).

A solution of the syrupy lactone (12.6 mg) in the minimal volume of water was mixed with a solution of brucine (26.2 mg) in ethanol (1 ml); the solution was kept for 5 days, the ethanol was evaporated, and the aqueous solution was extracted with ether (to remove the excess of brucine) and decolorized. On further standing, 15 mg of fine needles separated; these were recrystallized from aqueous ethanol; m.p. and m.m.p. (see next paragraph) 160–161° (dec.); X-ray powder diffraction data: 9.40 (s, 2), 6.71 (s, 3), 6.02 (s, 1), 4.85 (s), 4.60 (vw), 4.38 (w), 4.01 (w), 3.87 (m), and 3.73 (w).

Anal. Calc. for $C_{29}H_{36}N_2O_{11}$: C, 59.17; H, 6.16; N, 4.76. Found: C, 59.27; H, 5.92; N, 5.15.

From 6.3 mg of authentic L-iduronic acid[25] and 13.1 mg of brucine, 18 mg of the authentic brucinium salt of L-iduronic acid was obtained; this was recrystallized in the same way, m.p. 160–161° (dec.); X-ray powder diffraction data identical with those of the compound already described.

Hydrolysis of carboxyl-reduced derivative of heparin. — Partially desulfated, partially acetylated, carboxyl-reduced heparin[3] (100 mg), used in our previous work, was hydrolyzed by the modified, sulfuric acid method[13], as already described for the hydrolysis of the barium acid salt of heparin. A syrup (13.1 mg) was obtained after the removal of the sulfuric acid and the amino sugar component by the same procedure. Paper chromatography (solvent A) showed six spots, R_G 0.70 (D-glucuronic acid), 1.00 (D-glucose), 1.21 (L-iduronic acid), 1.58* (L-idose), 2.18 (D-glucuronolactone), and 3.23 (L-iduronolactone).

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DÉRIVÉS SULFONÉS, HALOGÉNÉS ET SOUFRÉS ASYMÉTRIQUES DU TRÉHALOSE. ÉTUDE DU 3,6-ANHYDRO-α,α-TRÉHALOSE*

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ABSTRACT

Treatment of trehalose with an equimolar proportion of methanesulfonyl or *p*-toluenesulfonyl chloride gave a mixture from which the monosulfonated derivatives at position 6 were isolated. Displacement of the *p*-tolylsulfonyloxy group with either I^- or SCN^- gave the corresponding iodo and thiocyano derivatives. Various procedures of reduction of the *p*-tolylsulfonyl as well as iodo or thiocyano derivatives always gave 3,6-anhydrotrehalose. The structure and conformation of this asymmetrical anhydride are discussed.

SOMMAIRE

L'action du chlorure de méthanesulfonyle ou du chlorure de *p*-toluènesulfonyle en proportions moléculaires sur le tréhalose, conduit à un mélange de composés d'où les dérivés monosulfonés en position 6 ont pu être isolés. Le déplacement par I^- ou SCN^- du *p*-toluènesulfonate conduit aux dérivés iodé et thiocyané correspondants. La réduction par différentes méthodes du *p*-toluènesulfonate aussi bien que des dérivés iodé et thiocyané conduit invariablement au 3,6-anhydrotréhalose. La structure et la conformation de cet anhydride asymétrique sont discutées.

INTRODUCTION

Le tréhalose, α-D-glucopyranosyl-α-D-glucopyranoside, possède une structure symétrique et la préparation de dérivés de ce disaccharide substitués sur un seul reste glucopyranosyle est de ce fait délicate. Même en utilisant des réactifs en quantités stoéchiométriques, les dérivés monosubstitués escomptés sont toujours accompagnés, en proportion variable, de leurs homologues provenant de l'attaque sur les deux cycles du tréhalose. Les dérivés asymétriques décrits jusqu'à maintenant sont : le tréhalose 6-phosphate[2], le 6-*O*-méthanesulfonyltréhalose[3], le 2-amino-2-désoxy-tréhalose[4], le 3-*ribo*-hexulotréhalose[5] et le 6-α-D-galactosyltréhalose[6].

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Dans le cadre de travaux sur la spécificité de la tréhalase du hanneton[1,6,7], nous avons tenté la préparation du 6-*O*-méthanesulfonyl-α,α-tréhalose (**1**) selon le mode opératoire décrit par Helferich[3]. Nous avons constaté que le produit décrit par cet auteur est en réalité un mélange d'au moins trois composants, ce qui nous a conduits à revoir les conditions de préparation de ce sulfonate. Nous basant sur ces résultats, nous avons pu de même obtenir le 6-*O*-*p*-toluènesulfonyl-α,α-tréhalose (**2**) à l'état pur. Nous pensions que ce dérivé nous aurait permis un accès aisé au 6-désoxy-α,α-tréhalose (**5**). Cependant l'application d'un certain nombre de méthodes classiques d'accès aux désoxysucres en partant d'un sulfonate[8] nous a conduits uniquement au 3,6-anhydrotréhalose (**6**).

Ce travail rapporte la préparation des 6-*O*-méthanesulfonyl- (**1**) et 6-*O*-*p*-toluènesulfonyl-α,α-tréhalose (**2**), des dérivés iodé (**3**) et thiocyané (**4**) correspondants, ainsi que l'application à ces composés des différentes méthodes de désulfonylation ou plus généralement de clivage réductif qui nous ont invariablement conduits au 3,6-anhydro-α,α-tréhalose (**6**).

RÉSULTATS ET DISCUSSION

La préparation du 6-*O*-méthanesulfonyl-α,α-tréhalose (**1**) d'après Helferich[3] nous a conduits à une substance amorphe dont les constantes physiques sont identiques à celles décrites par l'auteur. Le dérivé acetylé correspondant possède les mêmes constantes que celles décrites[3]. Par chromatographie sur plaque de gel de silice ou sur papier nous avons constaté que ces deux produits étaient en réalité constitués l'un et l'autre d'un mélange de trois composants que nous avons identifiés respectivement au tréhalose, à ses esters mono- et di-sulfonés, ainsi qu'aux dérivés acétylés correspondants dans le cas de l'hepta-*O*-acétyl-6-*O*-méthanesulfonyl-α,α-tréhalose précédemment décrit[3]. La séparation des dérivés acétylés par chromatographie préparative sur plaque de gel de silice nous a permis d'obtenir l'hepta-*O*-acétyl-6-*O*-méthanesulfonyl-α,α-tréhalose avec un rendement relativement faible. La désacétylation de ce composé par le méthylate de sodium[9] nous a conduits à un mélange contenant du 3,6-anhydrotréhalose (**6**). Ce résultat indique que, comme dans le cas des dérivés correspondants du D-glucopyranose, le sulfonate en position 6 du tréhalose est susceptible d'être déplacé aisément sous l'action d'un ion alcoxy en position 3.

Afin d'éviter cette transformation, le sulfonate (**1**) a été préparé directement par action du chlorure de méthanesulfonyle à −20° sur le tréhalose anhydre, avec un rendement de 6%. La structure de ce composé a été démontrée par méthylation selon Kuhn et Brossmer[10]. Le 6-*O*-*p*-toluènesulfonyltréhalose a été préparé d'une manière semblable.

Dans l'espoir d'obtenir le 6-désoxy-α,α-tréhalose (**5**), nous avons tenté d'appliquer au 6-*O*-*p*-toluènesulfonyl-α,α-tréhalose (**2**) la réduction par l'hydrure d'aluminium et de lithium[11], ainsi que le déplacement du groupe sulfonate par un agent nucléophile[8]. Devant la difficulté d'isolement à l'état pur du sulfonate (**2**), nous avons travaillé sur le mélange brut obtenu après sulfonation stoéchiométrique du tréhalose

suivie d'une acétylation. La teneur globale en esters sulfoniques de ce mélange est la même que si nous étions en présence d'un monosulfonate pur; nous avons donc déterminé la quantité du réactif en conséquence.

L'action de 2,5 moles d'hydrure de lithium et d'aluminium sur le mélange de sulfonates en solution dans le tétrahydrofuranne nous a conduits à un mélange de glucose, de tréhalose, de 3,6-anhydrotréhalose (**6**) et de 3,6:3′6′-dianhydro-α,α-tréhalose. Il semble par conséquent que les conditions alcalines utilisées dans cette réduction aient conduit exclusivement au déplacement du *p*-toluènesulfonate en C-6 par l'hydroxyle en position 3. Ce résultat n'est d'ailleurs pas totalement inattendu, puisque l'on sait par exemple que la réduction par l'hydrure de lithium et d'aluminium du monosulfonate du *cis*-3-hydroxyméthylcyclohexanol aboutit à la formation d'un cycle oxygéné à cinq chaînons[12]. On connaît par ailleurs la facilité de formation du 3,6-anhydro-D-glucose par action d'une base sur le 6-*O*-*p*-toluènesulfonate correspondant[13].

De façon à éviter la formation d'un ion alcoxy susceptible, comme dans le cas précédent, de conduire au déplacement d'un groupe polaire, nous avons pensé travailler sur les dérivés iodé ou thiocyané en 6 du tréhalose, dérivés dont la réduction fait intervenir des conditions beaucoup moins alcalines qui pourraient donc éviter la réaction parasite de formation d'un cycle oxygéné à cinq chaînons.

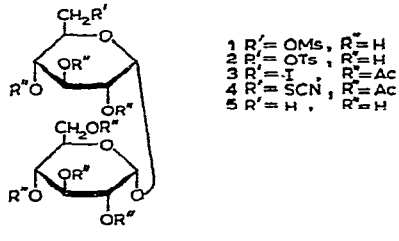
L'hepta-*O*-acétyl-6-désoxy-6-iodo-α,α-tréhalose (**3**) a été obtenu en partant, comme précédemment, du mélange brut contenant le sulfonate (**2**), par action de l'iodure de sodium dans l'anhydride acétique à reflux, suivie d'un purification par chromatographie. La réduction du mélange brut des acétates contenant l'halogénure (**3**), soit par l'hydrogène en présence de nickel Raney comme catalyseur et de quantités stoéchiométriques de diéthylamine, soit par l'hydrogène en présence de charbon palladié et de pyridine, a conduit dans les deux cas à un mélange de composés identifiés à l'octa-*O*-acétyl-α,α-tréhalose, à l'hexa-*O*-acétyl-3,6-anhydro-α,α-tréhalose (**6**) et au tétra-*O*-acétyl-3,6:3′6′-dianhydro-α,α-tréhalose. L'utilisation de quantités supérieures de diéthylamine provoque une désacétylation partielle du peracétyl-3,6-anhydro-α,α-tréhalose formé.

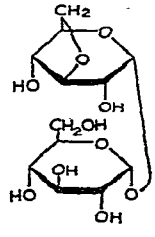
Nous avons alors tenté de réaliser cette réduction en milieu acide par la poudre de zinc dans l'acide acétique à 75% et en présence d'acide chloroplatinique comme catalyseur. Il semble que dans ces conditions le 6-désoxytréhalose se soit effectivement formé, mais ait subi immédiatement l'hydrolyse en glucose et 6-désoxyglucose comme l'a montré l'examen des produits de la réaction. Notons que le tréhalose n'est pas hydrolysé dans ces conditions. On sait que la stabilité des glycosides à l'hydrolyse acide décroît dans l'ordre : hexopyranosides > 6-désoxyhexopyranosides > pentopyranosides[14].

Un essai a été tenté en opérant sur le dérivé thiocyané en 6 du tréhalose (**4**) selon la méthode de Staněk et Tajmr[15]. L'action du thiocyanate de potassium sur le mélange brut contenant le 6-*O*-*p*-toluènesulfonyl-α,α-tréhalose dans la cyclohexanone à 130° conduit avec un bon rendement à un mélange de dérivés thiocyanés qui n'ont pu être séparés à l'état pur par chromatographie sur colonne de gel de

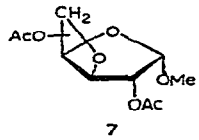
silice. La réduction de ce mélange par le nickel Raney dans le méthanol à reflux a conduit uniquement à un mélange dont les composants ont été identifiés à l'octa-*O*-acétyltréhalose, à l'hexa-*O*-acétyl-3,6-anhydro-α,α-tréhalose et au tétra-*O*-acétyl-3,6:3'6'-dianhydro-α,α-tréhalose.

Le 3,6-anhydro-α,α-tréhalose (**6**) obtenu au cours des réactions décrites précédemment a été caractérisé, après isolement chromatographique, par ses produits d'hydrolyse, glucose et 3,6-anhydroglucose, obtenus en quantité équimoléculaire, et par l'oxydation periodique selon Fleury et Lange[16]. Ces résultats ne peuvent suffire à indiquer la structure de ce composé, car la tension introduite dans le reste D-glucopyranosyle par la conformation d'un cycle à cinq chaînons du type 3,6-hydrofuranol est susceptible de provoquer un réarrangement du cycle glucopyranose en cycle glucofuranose[13], ou d'entraîner une anomérisation en forme β[17,18]. Il est donc indispensable de déterminer la configuration des restes glucosyle de l'anhydrotréhalose, et nous avons utilisé dans ce but le calcul de la rotation moléculaire selon les règles d'Hudson[19] modifiées par Wolfrom et Shafizadeh[20], de même que l'étude du spectre de résonance magnétique nucléaire (r.m.n.).

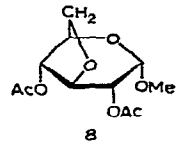




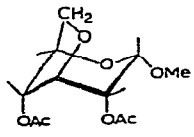
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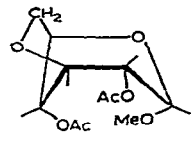
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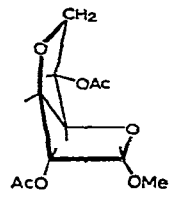
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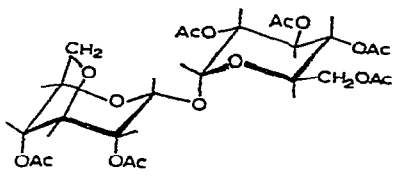
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11



12

Le pouvoir rotatoire moléculaire spécifique (M_D), calculé[19,20] sur la base de la rotation optique des méthyl 3,6-anhydro-D-glucofuranosides et pyranosides[13,21] est de +59.786 pour le 3,6-anhydro-α-D-glucofuranosyl α-D-glucopyranoside, et de +40.707 pour le 3,6-anhydro-α-D-glucopyranosyl-α-D-glucopyranoside. La valeur de

M_D trouvée expérimentalement pour le disaccharide (**6**) est de 42.921 (sous forme anhydre), ce qui est en faveur de la structure (**6**),

Le spectre de r.m.n. a été déterminé sur le dérivé peracétylé du 3,6-anhydro-α,α-tréhalose (**6**). L'interprétation a été réalisée en tenant compte des résultats obtenus avec les méthyl di-*O*-acétyl-3,6-anhydro-α-D-glucofuranoside (**7**) et -glucopyranoside (**8**), et avec l'octa-*O*-acétyl-α,α-tréhalose. Le spectre du composé (**7**) montre la présence de deux singulets peu différenciés à δ 2,1 p.p.m. (6 H). La seule conformation (**11**) est possible pour ce composé, et les deux acétates, sont magnétiquement équivalents. Le spectre du composé (**8**) montre dans la région des acétates deux singulets à δ 2,03 p.p.m. (3 H) et 2,16 p.p.m. (3 H). La différence de déplacement chimique entre ces deux singulets est de 7 Hz. On sait[18] que ce composé peut adopter deux conformations distinctes selon la nomenclature de Reeves[22] : a) chaise *1C* (**9**) et dans ce cas les deux acétates sont axiaux et magnétiquement équivalents; b) bateau *1B* (**10**) et les deux acétates présentent alors l'inéquivalence maximale. Le spectre est donc en faveur de la conformation *1B* (**10**) pour ce composé, bien que les interactions stériques semblent *a priori* peu favorables à cette conformation.

Comme on peut s'y attendre, le spectre de r.m.n. de l'octa-*O*-acétyl-α,α-tréhalose dont tous les acétates sont équatoriaux montre un multiplet centré à δ 2,05 p.p.m. (24 H) avec une différence maximale de déplacement chimique de 1 à 2 Hz pour l'ensemble des pics.

Le spectre de r.m.n. du 3,6-anhydro-α,α-tréhalose peracétylé montre deux multiplets distincts d'environ 8 Hz dans la région de vibration des acétates, δ 2,03 p.p.m. (12 H) et δ 2,16 p.p.m. (6 H). Ce comportement indique la présence de deux acétates axiaux et quatre acétates équatoriaux. L'absence de signaux des groupes acétates vers δ 2,1 p.p.m. élimine la forme furannique de la moitié 3,6-anhydro-glucose. Les signaux correspondant à 12 protons, visibles à champ plus élevé, sont imputables à la moitié D-glucopyranose du tréhalose. Les signaux correspondant à six protons, à plus bas champ, sont par conséquent uniquement attribuables à deux acétates axiaux de la partie 3,6-anhydro-D-glucopyranose. Contrairement au cas du méthyl 3,6-anhydro-α-D-glucopyranoside, il est probable qu'ici la conformation la plus stable du système bicyclique soit la conformation chaise *1C* (**12**), résultat confirmant la rotation optique de ce composé.

Le présent travail nous a permis de préparer un certain nombre de dérivés sulfonés, iodés, thiocyanés asymétriques, en C-6 du tréhalose, dont la réduction eût pu raisonnablement conduire au 6-désoxy-α,α-tréhalose. Cependant, tous les essais faisant intervenir des conditions même légèrement basiques ont invariablement conduit au 3,6-anhydro-α,α-tréhalose, du fait de la participation de l'hydroxyle en position 3 dans la réaction. Il est probable que la conformation du tréhalose favorise une telle réaction de déplacement.

Au cours de ce travail, nous avons eu connaissance des résultats de Birch[23] décrivant la préparation de l'hexa-*O*-acétyl-6,6'-didésoxy-α,α-tréhalose par hydrogénation catalytique de l'hexa-*O*-acétyl-6,6'-didésoxy-6,6'-diiodo-α,α-tréhalose en présence de nickel Raney et de diéthylamine. Après désacétylation, cet auteur pense

avoir obtenu uniquement le 6,6'-didésoxytréhalose en se fondant sur l'identification chromatographique des produits d'hydrolyse, comparativement à un témoin de glucose et de 2-désoxy-D-glucose. Ce résultat contraste avec les nôtres. Il est néanmoins possible que la conformation et par conséquent la réactivité du di-*O*-*p*-toluènesulfonyl-α,α-tréhalose diffère sensiblement de celle du 6-*O*-*p*-toluènesulfonyl-α,α-tréhalose.

PARTIE EXPÉRIMENTALE

Méthodes générales. — Les chromatographies sur papier d'identification ont été réalisées en système descendant sur feuilles « Schleicher Schull » 2043 B Mgl; les chromatographies préparatives, sur papier Whatman n° 3. Les solvants suivants ont été utilisés : alcool butylique–pyridine–eau (9:5:4) (solvant I) ; 2-propanol–alcool butylique–eau (7:1:2) (solvant II) ; acétate d'éthyle–pyridine–eau (10:4:3) (solvant III). Les taches ont été révélées par le nitrate d'argent suivant Trevelyan[24] pour les composés polyhydroxylés. Les chromatographies sur plaque ont été réalisées sur gel de silice Merck GF 254 avec les systèmes de solvants benzène–méthanol (96:4) et benzène–acétate d'éthyle (1:1). Les dérivés acétylés ont été révélés par le réactif à l'hydroxamate ferrique[25]. Les points de fusion ont été mesurés en tube capillaire sous microscope et sont corrigés. Les pouvoirs rotatoires ont été mesurés avec un polarimètre « Bourgogne 2 » Jobin et Yvon. Les spectres de r.m.n. ont été enregistrés sur un appareil « Varian A-60 » par M^me^ L. Alais. Les déplacements chimiques sont mesurés en p.p.m. (δ) à partir de la raie du tétraméthylsilane prise comme zéro de référence.

Le tréhalose, le 3,6-anhydrotréhalose et le 3,6:3',6'-dianhydrotréhalose ont été hydrolysés en ampoule scellée dans l'acide sulfurique 0.5M pendant 5 h au bain-marie bouillant. L'hydrolysat, neutralisé par le carbonate de strontium, est concentré sous pression réduite après filtration des sels minéraux. Le résidu, repris par le minimum d'eau est identifié par chromatographie sur papier dans les solvants I et II. La proportion relative des composants est appréciée après révélation du chromatogramme par le réactif au thymol–β-naphtylamine[26] et par élution des taches non révélées, par l'eau, suivie d'un dosage colorimétrique selon la technique de Nelson–Somogyi[27].

Les méthylations ont été réalisées selon la technique de Kuhn et Brossmer[10] sur les produits (10 mg) en solution dans la *N*,*N*-diméthylformamide (3 ml), par de l'iodure de méthyle (3 ml) en présence d'oxyde d'argent (0,5 g). Après un contact de 24 h dans l'obscurité sur agitateur magnétique, on filtre. Le précipité est lavé successivement avec de la *N*,*N*-diméthylformamide (2 ml), puis du chloroforme (30 ml) qui sont réunis au filtrat. Il se forme peu à peu un précipité blanc. Après séjour d'une heure à la glacière, on élimine le précipité par filtration. Le précipité est lavé au chloroforme. Le filtrat et l'extrait chloroformique sont évaporés à sec, sous vide. Le résidu est soumis de nouveau à la méthylation (4 fois) dans les mêmes conditions. Après la dernière opération, le filtrat est agité avec une solution de cyanure de potassium 0,5% (20 ml) pour enlever l'argent colloïdal en suspension, puis avec une solution de thiosulfate de sodium 50 mM pour éliminer l'iode libéré par la décom-

position de l'iodure de méthyle pendant l'opération. Après ces traitements, le filtrat est lavé avec de l'eau distillée, déshydraté par le sulfate de sodium anhydre et évaporé à sec, sous vide. Le résidu est soumis à une hydrolyse de 3 h par l'acide formique (2 ml) suivie, après évaporation de l'acide formique, d'une hydrolyse par l'acide sulfurique normal (3 ml) pendant 4 h. L'hydrolysat, après neutralisation, est soumis à une chromatographie ascendante sur papier Whatman n° 1 traité au préalable par une solution de chlorure d'ammonium 2% et séché à l'air libre; le solvant utilisé est un mélange de 2-méthylheptane–2-propanol–ammoniaque 10% (65:25:2). Un mélange témoin renfermant le 2,3,4,6-tétra-*O*-méthyl-D-glucose, les 2,3,4-; 2,3,6-; 2,4,6- et 3,4,6-tri-*O*-méthyl-D-glucose et le 2,3-di-*O*-méthyl-D-glucose est placé sur le même chromatogramme. Les oses méthylés sont révélés par le réactif à l'oxalate d'aniline de Partridge.

6-O-Méthanesulfonyl-α,α-tréhalose (**1**). (*a*) *Etude de l'hepta-O-acétyl-6-O-méthanesulfonyl-α,α-tréhalose préparé selon Helferich*[3]. — Ce composé préparé selon la méthode décrite[3] et dont les constantes sont identiques à celles données par les auteurs a été chromatographié sur une plaque de gel de silice préparative selon la technique de Tate et Bishop[25] et a conduit à trois substances. La désacétylation de ces dérivés peracétylés, chromatographiquement purs, a été réalisée selon la méthode de Zemplen[9]. Par chromatographie sur papier dans les systèmes de solvants I et II, on observe respectivement pour ces trois composés une tache de R_G 0,57 (I) et 0,64 (II) identique au tréhalose témoin et deux taches de moindre importance de R_G 1,22 (I), 0,97 (II) correspondant au 6-*O*-méthanesulfonyl-α,α-tréhalose et 1,52 (I) et 1,30 (II) correspondant au 6,6'-di-*O*-méthanesulfonyl-α,α-tréhalose. On remarque avec le second composant une tache de faible intensité migrant comme le 3,6-anhydro-α,α-tréhalose, R_G 1,09 (I) et 1,05 (II).

(*b*) *6-O-Méthanesulfonyl-α,α-tréhalose* (**1**). — A une solution refroidie à −20° de tréhalose anhydre (10 g) (29 mmoles) dans la pyridine (40 ml) on ajoute goutte à goutte en 20 min et sous agitation magnétique du chlorure de méthanesulfonyle (3,5 ml; 45 mmoles) également refroidi à −20°. Après une nuit à −20°, on laisse revenir à la température ambiante, puis on ajoute un volume de chloroforme égal à celui de la solution. Il se forme un précipité gélatineux qui est lavé à plusieurs reprises par le chloroforme, jusqu'à élimination de la pyridine. Le précipité est repris par l'eau et la solution filtrée est évaporée jusqu'à consistance sirupeuse. Ce sirop se révèle, à la chromatographie, être composé de trois constituants : tréhalose, 6-*O*-méthanesulfonyl-α,α-tréhalose et des traces de 6,6'-di-*O*-méthanesulfonyl-α,α-tréhalose (la majorité de ce dernier composé est éliminée par l'extraction au chloroforme). Le 6-*O*-méthanesulfonyl-α,α-tréhalose est purifié par trois chromatographies successives sur papier Whatman n° 3 avec un système de solvants non alcalins (2-propanol–méthyléthylcétone–acétate d'éthyle–alcool butylique–eau, 6:5:3:2:5). Les extraits aqueux sont lyophilisés et conduisent à 735 mg (5,98%) d'une poudre blanche; $[\alpha]_D^{20}$ +131° (*c* 0,306, eau); R_G 1,22 (solvant I); 0,97 (solvant II); 1,21 (solvant III).

Anal. : Calc. pour $C_{13}H_{24}O_{13}S$: C, 37,17; H, 5,75; S, 7,62. Trouvé : C, 37,20; H, 5,71; S, 7,39.

6-O-p-Toluènesulfonyl-α,α-tréhalose (**2**). — A une solution de tréhalose anhydre (5 g, 14,5 mmoles) dans la pyridine (20 ml) refroidie à une température inférieure à 0°, on ajoute une solution également refroidie de chlorure de *p*-toluènesulfonyle (3,1 g, 16 mmoles) dans la pyridine (20 ml). On laisse une nuit à la glacière. La solution est amenée ensuite à pH 4 avec de l'acide sulfurique M et extraite trois fois avec du chloroforme (30 ml). L'extrait chloroformique, lavé jusqu'à réaction neutre et amené à sec sous pression réduite (1,120 g), contient du 6,6'-di-*O-p*-toluènesulfonyl-α,α-tréhalose et quelques traces d'un homologue supérieur. Le 6-*O-p*-toluènesulfonyl-tréhalose et le tréhalose restent dans la phase aqueuse. Cette dernière est neutralisée par le carbonate de strontium et extraite plusieurs fois par le butanol. L'extrait butanolique est évaporé sous pression réduite et la pyridine résiduelle est chassée par un mélange azéotrope chloroforme–méthanol. Une solution aqueuse du résidu est adsorbée sur une colonne de poudre de cellulose Whatman de 45 cm de haut, 3,5 cm de diamètre avec le solvant II comme éluant. On recueille des fractions de 15 ml. Les fractions donnant une réaction positive à l'anthrone sont examinées par chromatographie sur papier. Les fractions 32 à 40 contiennent du 6,6'-di-*O-p*-toluènesulfonyl-α,α-tréhalose ainsi que du 6-*O-p*-toluènesulfonyl-α,α-tréhalose. Les fractions 40 à 62 contiennent seulement le 6-*O-p*-toluènesulfonyl-α,α-tréhalose. Elles sont rassemblées et évaporées à température inférieure à 30°. Le résidu est repris par l'eau, et la solution est filtrée et lyophilisée (434 mg, 5,98%); $[\alpha]_D^{20}$ +125° (*c* 0,22, eau); R_G 1,97 (solvant I); 1,66 (solvant II); 2,02 (solvant III).

Anal. : Calc. pour $C_{19}H_{28}O_{13}S$: C, 45,96; H, 5,67; S, 6,45. Trouvé : C, 45,81; H, 5,63; S, 6,52.

3,6-Anhydro-α,α-tréhalose (**6**). — Le tréhalose anhydre (5 g, 14,5 mmoles) est dissous dans la pyridine (20 ml) et placé à −10°. On ajoute goutte à goutte une solution également refroidie de chlorure de *p*-toluènesulfonyle (3,1 g, 16 mmoles) dans la pyridine (20 ml) tout en agitant. Après une nuit à la glacière, on laisse revenir à la température ambiante et on ajoute de l'anhydride acétique (30 ml) en refroidissant. Après 24 h de contact, on ajoute quelques gouttes d'eau glacée, puis on verse le tout dans un récipient contenant 3 l d'eau glacée. Il se forme peu à peu un précipité qu'on recueille par filtration. Après deux recristallisations dans l'alcool absolu bouillant, on obtient des cristaux en fines aiguilles (8,532 g) correspondant à un mélange de dérivés sulfonés et acétylés du tréhalose. La chromatographie sur plaque de gel de silice, suivie d'une révélation selon Tate et Bishop[25] permet d'estimer que ce mélange contient environ 75% du *p*-toluènesulfonate **2** acétylé.

Le mélange de dérivés acétylés (1 g) est dissous dans du tétrahydrofuranne (30 ml), puis additionné par petites fractions, tout en refroidissant, d'hydrure de lithium et d'aluminium (0,5 g) (environ 2,5 équiv. molaires). On porte à l'ébullition à reflux pendant 30 min. Après refroidissement la réaction est arrêtée par addition d'acétate d'éthyle. On filtre sur Hyflosupercel et lave le précipité d'alumine à l'eau. Le filtrat est évaporé sous pression réduite jusqu'à consistance sirupeuse. L'opération répétée trois fois donne 1,392 g d'un mélange qui, repris par l'eau, est déminéralisé par passages successifs sur des colonnes d'Amberlite IR-120 (H^+), puis IRA-400

(OH^-). Après évaporation sous pression réduite et dessiccation, on obtient 730 mg de produit. La chromatographie sur papier indique la présence de trois composants, identifiés au tréhalose, au 3,6-anhydro-α,α-tréhalose et au 3,6:3′,6′-dianhydro-α,α-tréhalose. On remarque également la présence d'une trace de glucose. La séparation sur colonne de charbon–Célite (1:1) de ces composants, suivie d'une hydrolyse acide dans les conditions décrites confirme leur structure.

Le mélange sirupeux (5,1 g) obtenu à partir de onze préparations consécutives, a donné, après passage sur colonnes échangeuses d'ions, 3,362 g de produit qui ont été purifiés sur trois colonnes de 50 g chacune d'un mélange charbon–Célite à parties égales. L'élution a été réalisée avec un gradient obtenu en faisant arriver dans un flacon mélangeur à volume constant contenant 800 ml d'eau, de l'alcool à 10% contenu dans un réservoir de 2 litres, ce dispositif conduisant à un gradient exponentiel convexe. L'éluat a été recueilli par fractions de 25 ml, après être passé dans la cellule d'un polarimètre automatique (les déviations sont enregistrées sur un appareil Graphispot). Les différentes fractions ont été examinées par chromatographie sur papier. Le premier pic renfermait du glucose, ensuite venait un mélange de glucose et de tréhalose; le deuxième pic renfermait du tréhalose; le troisième uniquement le 3,6-anhydro-α,α-tréhalose. Les fractions correspondant au troisième pic ont été groupées et évaporées sous pression réduite. A partir de trois colonnes de charbon, nous avons obtenu 708 mg (10,7%) d'un composé cristallin qui, redissous dans le minimum d'alcool absolu à chaud, donnent par refroidissement le 3,6-anhydro-α,α-tréhalose sous forme de longues aiguilles qui correspondent à un hydrate avec 1,5 molécule d'eau; p.f. 95–96°, $[\alpha]_D^{20}$ +115° (*c* 0,5, eau).

Anal. : Calc. pour $C_{12}H_{22}O_{11} \cdot 1{,}5\ H_2O$: C, 41,02; H, 6,58. Trouvé : C, 40,95; H, 6,48.

L'oxydation periodique du 3,6-anhydro-tréhalose a été effectuée comparativement à celle du tréhalose et du saccharose en oxydant à la glacière le disaccharide (0,2 mmoles) par l'acide periodique (2 mmoles). L'acide periodique consommé et l'acide formique libéré ont été dosés d'après Fleury et Lange[16]. Acide periodique consommé : 0,347, 0,447, 0,796, 1,720, 2,039, 2,080 et 2,040; acide formique libéré : 0, 0,042, 0,21, 0,42, 0,62, 0,94, et 0,96 en 30 min, 1, 4, 24 et 48 h, et 4 et 15 jours, respectivement. En 15 jours le tréhalose et le saccharose on consommé 3,999, 3,208 moles d'acide periodique, respectivement, et libéré 2 et 0,94 moles d'acide formique respectivement.

Hepta-O-acétyl-6-désoxy-6-iodo-α,α-tréhalose (3). — Le mélange de sulfonates acétylés (10 g) est dissous dans l'anhydride acétique (120 ml) et additionné d'iodure de sodium sec (3,80 g). Après une ébullition à reflux de 2,5 h, le mélange est refroidi et versé dans deux parties d'eau glacée. Une huile précipite, qui est lavée à l'eau froide à plusieurs reprises. On la reprend par le méthanol à chaud; après traitement de la solution méthanolique par le charbon animal, on obtient de fines aiguilles par concentration du filtrat (6,54 g). La chromatographie sur plaque de gel de silice indique la présence de trois composants. Une chromatographie sur colonne de gel de silice équilibrée dans le solvant benzène–éther (7:3) permet d'isoler par élution

par un mélange benzène–éther (63:35) l'halogénure asymétrique **3** qui est cristallisé dans l'éthanol; p.f. 80–82°, $[\alpha]_D^{29}$ +121,5° (*c* 0,93, chloroforme).

Anal. : Calc. pour $C_{26}H_{35}O_{17}I$: C, 41,82; H, 4,69; I, 17,02. Trouvé : C, 41,85; H, 4,56; I, 17,19.

Essai d'obtention du 6-désoxy-α,α-tréhalose. (a) *Par réduction du composé* **3**. — Les essais ont été réalisés sur le mélange brut des acétates contenant l'hepta-*O*-acétyl-6-désoxy-6-iodo-α,α-tréhalose. Les dérivés iodés et acétylés du tréhalose (1 g) sont dissous dans du méthanol (30 ml) additionné de diéthylamine (0,15 ml, env. 1,4 équiv. molaire). L'hydrogénation est réalisée à température ordinaire en présence de nickel Raney (0.5 g). Quand la réaction est terminée (consommation de 30 ml d'hydrogène en 1 h), la suspension est filtrée et le filtrat concentré sous pression réduite. Le résidu est repris par le chloroforme et lavé à l'eau. L'extrait chloroformique est concentré sous vide. Après cristallisation dans l'éthanol, nous obtenons 659 mg d'un mélange de produits qui ont été identifiés par chromatographie sur plaque de gel de silice, comparativement à des échantillons authentiques, respectivement à l'octa-*O*-acétyl-α,α-tréhalose, à l'hexa-*O*-acétyl-3,6-anhydro-α,α-tréhalose et au tétra-*O*-acétyl-3,6:3′,6′-dianhydro-α,α-tréhalose. Ce mélange est identique à celui obtenu après acétylation du produit brut résultant de la réduction par l'hydrure de lithium et d'aluminium du mélange brut contenant le sulfonate **2**. Par désacétylation selon Zemplen[9] et hydrolyse du mélange précédent par l'acide sulfurique 0.5M pendant 4 h au bain-marie bouillant on obtient uniquement le D-glucose et le 3,6-anhydro-D-glucose, comme l'indique la chromatographie sur papier dans les solvants I, II et III comparativement à des échantillons authentiques.

Le mélange contenant l'halogénure **3** (1 g) est dissous dans le méthanol (40 ml) et additionné de pyridine (0.1 ml; 1 équiv. molaire) et de charbon palladié à 10% (0,5 g). L'hydrogénation est réalisée à 60° pendant 4 heures. Le catalyseur est éliminé par filtration et la solution évaporée jusqu'à consistance sirupeuse. On reprend par le chloroforme (50 ml), lave avec une solution à 1% de thiosulfate de sodium puis par l'eau distillée. La solution chloroformique séchée sur sulfate de sodium anhydre est amenée à sec sous pression réduite. Le résidu est recristallisé dans l'éthanol bouillant. La chromatographie sur gel de silice montre que ces cristaux consistent en un mélange de tréhalose, de mono- et de dianhydro-tréhalose peracétylés.

Les dérivés iodés et acétylés (1 g) sont dissous dans l'acide acétique à 75% (20 ml) et additionnés d'une trace d'acide chloroplatinique. Le mélange est porté à 80° dans un bain-marie avec agitation magnétique et additionné de poudre de zinc (1,5 g) par petites fractions sur une durée de 15 min. Après filtration à chaud et concentration sous pression réduite, le résidu est repris par le chloroforme et la solution chloroformique lavée avec une solution d'hydrogèno-carbonate de soude de façon à éliminer l'acide acétique résiduel. Après évaporation et recristallisation dans l'éthanol, le produit est désacétylé de la manière habituelle. La chromatographie sur papier révèle la présence de glucose, de quinovose, de tréhalose ainsi que de traces d'un composé correspondant probablement au désoxytréhalose recherché.

(b) *Par réduction du dérivé thiocyané du tréhalose.* — Les dérivés tosylés et acétylés

(7 g) sont dissous dans la cyclohexanone (100 ml), additionnés de thiocyanate de potassium (4 g) et portés 2 h à 130° tout en agitant. Après refroidissement, le mélange réactionnel est additionné d'eau (200 ml) et extrait par le chloroforme (200 ml). La phase chloroforme–cyclohexanone, après lavage à l'eau, est séchée sur sulfate de sodium et évaporée à sec. Le mélange de dérivés acétylés et thiocyanés cristallise dans le méthanol (5,005 g). Une chromatographie sur plaque montre que la transformation des dérivés tosylés a été totale. Une partie du mélange (1 g) est alors dissoute dans 200 ml de méthanol et portée à reflux avec du nickel Raney(2,6 g) pendant 6 h. Le nickel est éliminé par filtration et le filtrat, amené à sec sous pression réduite et repris par le méthanol, fournit 640 mg de cristaux dont la chromatographie sur plaque de gel de silice montre qu'il s'agit d'un mélange de tréhalose, de 3,6-anhydro-α,α-tréhalose et de 3,6:3′,6′-dianhydro-α,α-tréhalose peracétylés. La désacétylation suivie d'une hydrolyse acide de ce mélange brut conduit uniquement au D-glucose et au 3,6-anhydro-D-glucose, comme le montre la chromatographie sur papier dans les solvants I, II, III de cette substance, comparativement à des échantillons authentiques.

Octa-O-acétyl-α,α-tréhalose. — Le tréhalose dihydrate (500 mg) est dissous dans l'anhydride acétique (25 ml) et additionné de carbonate de sodium anhydre (250 mg). Après une ébullition de 15 min, la solution est refroidie et le passage d'un courant d'air comprimé provoque une cristallisation en masse. Les cristaux sont alors repris par le minimum d'un mélange eau–éthanol (1:3). Après une nuit, on obtient des cristaux qui, examinés dans l'eau, se présentent au microscope sous forme d'hexagones réguliers. Par dissolution de ces cristaux dans l'éthanol et refroidissement, il se forme d'abord des hexagones puis la majorité des cristaux se convertit en aiguilles très fines qui, avec le temps, évoluent en longues aiguilles. P.f. 99° (hexagones), 76–78° (aiguilles); lit.[28] : p.f. 70–75°.

Anal. : Calc. pour $C_{28}H_{38}O_{19}$: C, 49,66; H, 5,60. Trouvé : C, 49,59; H, 5,48.

Le tréhalose anhydre (2 g) est dissous dans la pyridine également anhydre (25 ml). On ajoute de l'anhydride acétique (12 ml). Après 24 h de contact, le mélange est versé dans l'eau glacée. Le précipité qui se forme est recueilli et dissous dans le méthanol. Après recristallisation dans le méthanol, on obtient des aiguilles qui fondent à 78°.

Hexa-O-acétyl-3,6-anhydro-α,α-tréhalose (**12**). — La solution de 3,6-anhydro-α,α-tréhalose (1,5 H_2O) (100 mg) dans la pyridine (5 ml) est refroidie à −4° et additionnée d'anhydride acétique (4 ml) en évitant tout échauffement. Après 24 h de contact, le mélange réactionnel est additionné d'une solution glacée de sulfate acide de potassium à 5% et extrait plusieurs fois par le chloroforme. L'ensemble des extraits chloroformiques est lavé par une solution d'hydrogénocarbonate de sodium à saturation, puis par l'eau. Après séchage sur sulfate de sodium, la solution est amenée à sec sous pression réduite. Le résidu cristallise dans l'alcool absolu (144 mg), p.f. 171–172°, $[\alpha]_D^{20}$ +154° (*c* 0,25, chloroforme).

Anal. : Calc. $C_{24}H_{32}O_{16}$: C, 50,01; H, 5,60. Trouvé : C, 50,29; H, 5,65.

Méthyl 2,4-di-O-acétyl-3,6-anhydro-α-D-glucopyranoside (**8**). — Le méthyl 3,6-anhydro-α-D-glucopyranoside est acétylé selon la technique précédente. Le

peracétate est obtenu sous forme d'aiguilles par recristallisation dans le méthanol, p.f. 134–135°, $[\alpha]_D^{24}$ +101,6° (*c* 1,2, chloroforme).

Anal. : Calc. pour $C_{11}H_{16}O_7$: C, 50,77; H, 6,20; $CH_3(CO)$, 11,53. Trouvé : C, 50,76; H, 6,30; $CH_3(CO)$, 11,75.

Méthyl 2,5-di-O-acétyl-3,6-anhydro-α-D-glucofuranoside (7). — L'acétylation du méthyl-3,6-anhydro-α-D-glucofuranoside est réalisée selon la méthode précédente et donne une huile, $eb._{10-2}$ 100–105°, n_D^{23} 1,4620, $[\alpha]_D^{24}$ +235,2° (*c* 1,7, chloroforme).

Anal.: Calc. pour $C_{11}H_{16}O_7$: C, 50,77; H, 6.20; $CH_3(CO)$, 11.53. Trouvé: C, 51.07; H, 6.36; $CH_3(CO)$, 12.04.

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CONFORMATION OF ACYCLIC DERIVATIVES OF SUGARS. CONFORMATIONS OF PERACETYLATED ALDOSE DITHIOACETALS IN SOLUTION* †

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ABSTRACT

The peracetylated diethyl dithioacetals of D-ribose, D-arabinose, D-xylose, D-lyxose, L-rhamnose, and L-fucose were studied in chloroform-*d* solution by n.m.r. spectroscopy at 100 MHz. Signals of all protons were assigned, assignments were verified by spin-decoupling, and first-order coupling-constants were determined. For the D-arabinose, D-lyxose, L-rhamnose, and L-fucose derivatives, the coupling constants observed accord with a favored conformation in which the chain of carbon atoms is fully extended in a planar, zig-zag arrangement. The D-ribose and D-xylose derivatives, which, in a conformation having the carbon atoms in a planar, zig-zag arrangement, would have a pair of parallel, eclipsed acetoxyl groups at C-2 and C-4, give coupling data completely inconsistent with such an extended, planar formulation. The data indicate that the favored conformation of the D-ribose and D-xylose derivatives is a "sickle" arrangement derived from the planar, zig-zag conformation by rotation (about the C-3–C-4 bond in the D-ribose derivative, and about the C-2–C-3 bond in the D-xylose derivative) to rotamers in which the steric interaction between substituents at C-2 and C-4 is relieved.

INTRODUCTION

In the first paper in this series[2], it was shown that the vicinal spin-couplings observed in the n.m.r. spectra of 2-(D-*arabino*-tetrahydroxybutyl)quinoxaline and its tetraacetate, in deuterium oxide and chloroform-*d*, respectively, are consistent with a favored conformation having an extended, planar, zig-zag arrangement of carbon atoms in the polyhydroxyalkyl chain, corresponding to achievement of minimum, nonbonded interactions between the sets of small–medium–large groups along each carbon–carbon bond. In a detailed, comparative study[1], by n.m.r. spectroscopy, of the conformations of the polyhydroxyalkyl chains of various sugar

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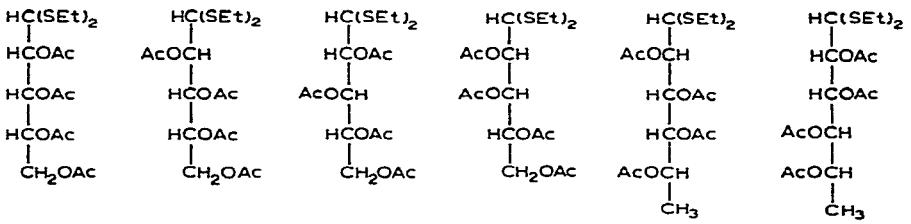
TABLE I

CHEMICAL-SHIFT DATA FOR ACETYLATED ALDOSE DIETHYL DITHIOACETALS

Compound	*Configuration*	*Chemical shifts* (τ) *in chloroform-d*									
		H-1	*H-2*	*H-3*	*H-4*	*H-5*[a]	*H-5′*	*H-6*	CH_2[b]	CH_3[b]	*Acetyl groups*
1	*ribo*	6.01	4.67	4.37	4.62	5.55	5.89		7.32	8.76, 8.78	7.89, 7.95, 7.97, 8.00
2	*arabino*	6.09	4.70	4.28	4.87	5.70	5.98		7.34	8.77, 8.80	7.92, 7.93, 7.99, 8.00
3	*xylo*	6.02	4.65	4.26	4.62	5.67	5.99		7.34	8.74, 8.80	7.96 (2), 7.98, 8.02
4	*lyxo*	6.11	4.65	4.35	4.61	5.73	6.10		7.35	8.76, 8.77	7.89, 7.95 (2), 7.99
5	*galacto*	6.16	4.81	4.19	4.93	5.05		8.87	7.36	8.77, 8.80	7.88, 7.91, (2), 7.97
6	*manno*	6.13	4.69	4.20	4.73	5.09		8.81	7.35	8.76, 8.77	7.94, 7.95, 7.96, 7.99

[a]When two protons are present at C-5, the one resonating at lower field is designated H-5, and that resonating at higher field is designated H-5′. [b]Of the ethyl groups.

phenylosotriazoles, it was shown that the measurements are again consistent with the extended, planar, zig-zag conformation, except where such an arrangement would lead to a parallel 1,3-interaction of hydroxyl groups. In the latter situation, the n.m.r. spectral evidence indicated that the chain adopts a conformation in which the 1,3-interaction is relieved by rotation about one carbon–carbon bond to a rotamer having a gauche interaction between substituents on vicinal carbon atoms, but no parallel 1,3-interaction between hydroxyl groups. In order to assess the possible generality of these results in other systems containing polysubstituted, acyclic chains, we have examined a number of series of acyclic derivatives of sugars, with representative configurational isomers in each series. The present report describes the conformations in solution, as revealed by n.m.r. spectroscopy, of tetra-*O*-acetyl-D-ribose diethyl dithioacetal (**1**), tetra-*O*-acetyl-D-arabinose diethyl dithioacetal (**2**), tetra-*O*-



acetyl-D-xylose diethyl dithioacetal (**3**), tetra-*O*-acetyl-D-lyxose diethyl dithioacetal (**4**), tetra-*O*-acetyl-L-fucose diethyl dithioacetal (**5**), and tetra-*O*-acetyl-L-rhamnose diethyl dithioacetal (**6**). The data accord well with formulation of compounds **1** to **6** in conformations that accord with the principles set out in the previous studies[1,2].

SPECTRAL DATA

The 100-MHz n.m.r. spectrum of each acetylated dithioacetal (**1**–**6**) was measured at ~28° in chloroform-*d* containing 5% of tetramethylsilane. Spectral analysis was effected or verified, as necessary, by spin decoupling. The measured chemical shifts are given in Table I, and the first-order coupling constants, in Table II. The low-field portion of the spectrum of the D-arabinose derivative (**2**) is shown in Fig. 1, and that of the L-rhamnose derivative (**6**) is shown in Fig. 2, as representative spectra of the pentose and 6-deoxyhexose derivatives, respectively.

In all six examples, the two ethylthio groups are nonequivalent and give closely spaced, overlapping triplets near τ 8.78 for the methyl protons of the ethyl groups, exhibiting a vicinal spin-coupling of 7.1 $\pm$0.2 Hz. The methylene protons give rise to a multiplet near τ 7.34; the multiplicity of this signal is frequently greater than that of a pair of overlapping quartets, suggesting appreciable nonequivalence between individual protons of the methylene groups. Signals of the acetyl-methyl protons are

observed between τ 7.88 and 8.02. Protons on the sugar chain give signals whose multiplicities and chemical shifts are markedly dependent on configuration.

TABLE II

FIRST-ORDER COUPLING-CONSTANTS FOR ACETYLATED ALDOSE DIETHYL DITHIOACETALS

Compound	*Configuration*	*Coupling constants, Hz*							
		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$^aJ_{4,5}$	$^aJ_{4,5'}$	$J_{5,5'}$	$J_{5,6}$	$J_{CH_2CH_3}$
1	*ribo*	6.2	5.7	3.6	3.1	7.7	12.0		7.3
2	*arabino*	8.3	2.8	7.9	2.9	6.0	12.3		6.9
3	*xylo*	5.2	5.9	4.2	4.3	6.6	11.8		7.3
4	*lyxo*	4.2	7.9	2.0	4.9	7.6	11.5		7.1
5	*galacto*	8.2	1.8	9.1	2.2			6.5	7.2
6	*manno*	4.7	7.7	1.8	8.4			6.3	7.3

[a]See footnote *a* of Table I.

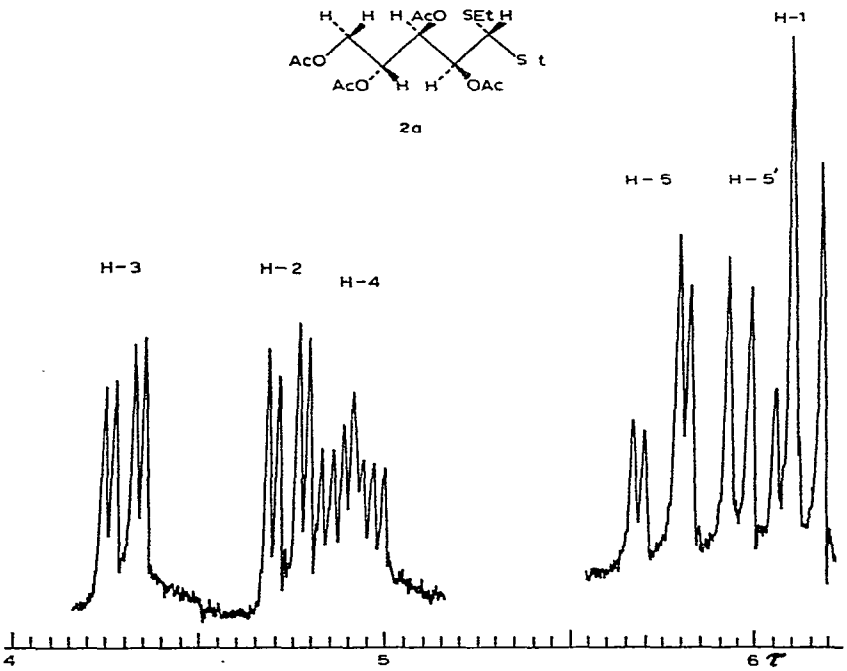


Fig. 1. The low-field portion of the n.m.r. spectrum of tetra-*O*-acetyl-D-arabinose diethyl dithioacetal (2) in chloroform-*d* at 100 MHz.

In each example, the H-1 signal is observed at τ 6.0–6.1 as a doublet whose spacing gives $J_{1,2}$. The position of this signal, at higher field than all of the other methine-proton signals, is attributable to the presence of the two sulfur atoms attached to C-1. It is known[3,4] that replacement of a single oxygen atom at C-1 of a sugar derivative by sulfur causes the H-1 signal to be shifted upfield. In the case of the dithioacetals, the H-1 signal appears at considerably higher field than in the

1,1-dioxygenated analogs[5]. In the 6-deoxyhexose derivatives (compare Fig. 2) the H-1 doublet is separated from the other signals. For the pentose derivatives, the H-1 doublet overlaps the signal of one of the C-5 protons (compare Fig. 1).

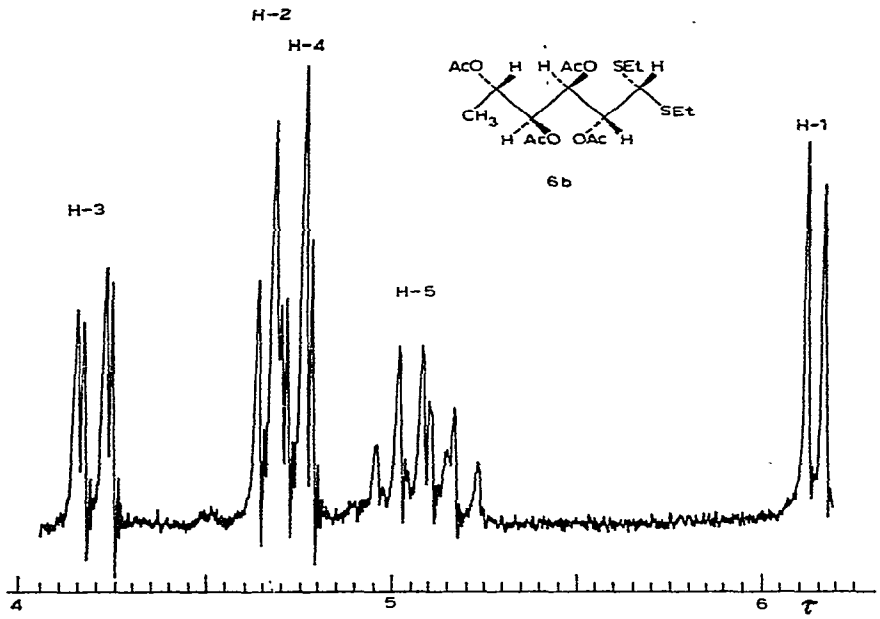


Fig. 2. The low-field portion of the n.m.r. spectrum of tetra-*O*-acetyl-L-rhamnose diethyl dithioacetal (6) in chloroform-*d* at 100 MHz.

The lowest-field signal in all six examples is that of H-3, observed as a quartet whose spacings correspond to $J_{2,3}$ and $J_{3,4}$. At somewhat higher field, the pentose derivatives (**1–4**) show a 2-proton multiplet arising from the superposed, or closely adjacent, signals of H-2 and H-4. The 6-deoxyhexose derivatives (**5** and **6**) show the H-5 signal, also, in this region. At somewhat higher field (τ 5.5–5.7), the pentose derivatives **1–4** exhibit a quartet, separated from the other signals, for one of the C-5 protons (H-5). This signal is the B portion of an ABX system and its spacing gives the vicinal ($J_{5,5'}$) coupling and the value of $J_{4,5}$. At still higher field (τ 5.9–6.1), the other C-5 proton (H-5′) gives a quartet as the A portion of the ABX system, and its narrow spacing gives $J_{4,5'}$. The latter quartet overlaps the H-1 doublet. The 6-deoxyhexoses show the H-6 signal as a 3-proton doublet overlapping the signals for the methyl portion of the ethylthio groups.

In the spectra of **1–4**, the chemical-shift difference between each pair of coupled protons is substantial, and conditions for virtual coupling[6] are not present. The first-order analysis is, therefore, justified, and the measured spacings should be very close to the true coupling-constants. The values of $J_{1,2}$, $J_{4,5}$, and $J_{4,5'}$ are evident by direct inspection; the values of $J_{2,3}$ and $J_{3,4}$ measured from the H-3 signal may be assigned specifically by analysis of the multiplet for H-2 and H-4. The H-2 signal is

observed as a quartet (apparent triplet in the case of **1** and **3**) having the $J_{1,2}$ spacing and also the $J_{2,3}$ spacing. The latter is identical with one of the two spacings of the H-3 signal. The H-4 signal is observed as a multiplet whose width ($J_{3,4}+J_{4,5}+J_{4,5'}$) permits $J_{3,4}$ to be determined; it is identical with the other spacing of the H-3 signal. The assignments were further confirmed by irradiating, in turn, the H-3 signal, the H-5 signal, and the H-5′ and H-1 signals, and observing the appropriate collapse of signals.

The spectra of the 6-deoxyhexose derivatives **5** and **6** were analyzed similarly for H-1, 2, and 3, to give $J_{1,2}$, $J_{2,3}$, and $J_{3,4}$. The methylene-proton signal of the ethylthio groups was irradiated to collapse the corresponding methyl-proton signals, whereupon the H-6 doublet was observed clearly; its spacing gives $J_{5,6}$. Irradiation of the H-6 signal caused collapse of the H-5 signal into a doublet whose spacing gives $J_{4,5}$.

DISCUSSION

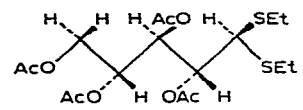
Assuming that torsional barriers to rotation about all bonds are small enough to allow free interconversion of rotamers at the temperature (~28°) of the measurements, the energetically favored conformer or conformers will preponderate in the statistical distribution of molecular shapes, in accord with the dictates of classical thermodynamics. By further assuming that vicinal, antiparallel protons show spin–spin coupling of 8–9 Hz and that vicinal, gauche protons having a dihedral angle of ~60° are coupled by[7,8] 3–4 Hz, it is possible to interpret the observed coupling data in terms of predicted, favored conformations.

Tetra-O-*acetyl*-D-*arabinose diethyl dithioacetal* (**2**). — The coupling data for **2** accord entirely with a favored conformation (**2a**) having an essentially planar, zig-zag backbone composed of carbon atoms 1–5, O-5, and a sulfur atom at C-1, with the C-1–C-2 rotamer being that which brings the other sulfur atom at C-1 antiparallel to O-2. The observed $J_{1,2}$ and $J_{3,4}$ values (8.3 and 7.9 Hz, respectively) demonstrate the preponderance of those rotamers having H-1 and H-2, and H-3 and H-4, antiparallel. The $J_{2,3}$ value (2.8 Hz) accords with conformation **2a**; the other possible conformer having a gauche relationship between H-2 and H-3 would generate an unfavorable 1,3-interaction[1], between O-2 and O-4. The fact that the larger of the 4,5-couplings ($J_{4,5'}$) is only 6.0 Hz suggests that an appreciable contribution to the rotamer population may be made by that rotamer having O-5 bisecting the angle of O-4 and C-3.

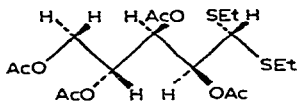
The conformation **2a** corresponds to maximum relief of steric interactions between small–medium–large sets of groups along each carbon–carbon bond, and does not introduce any parallel 1,3-interactions of substituents.

Tetra-O-*acetyl*-L-*fucose diethyl dithioacetal* (**5**). — This compound is homomorphous with **2** at C-2, C-3, and C-4. The coupling data support a favored conformation (**5a**) having an essentially planar, zig-zag chain of carbon atoms similar to that in **2a**. The antiparallel disposition of H-1 and H-2, and H-3 and H-4, is evident

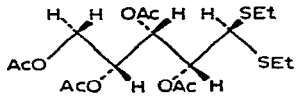
from the respective $J_{1,2}$ and $J_{3,4}$ couplings (8.2 and 9.1 Hz), and the small values of $J_{2,3}$ and $J_{4,5}$ indicate the gauche arrangement of H-2 and H-3, and H-4 and H-5. The alternative, gauche conformers at C-2–C-3 and C-4–C-5 would, in each case, lead to the unfavorable 1,3-interaction of acetoxyl groups.



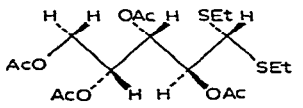
1a



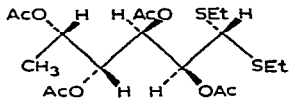
2a



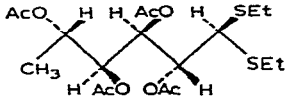
3a



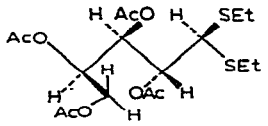
4a



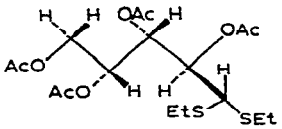
5a



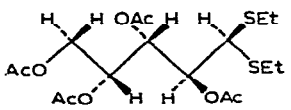
6a



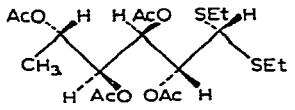
1b



3b



4b



6b

The particularly small value (1.8 Hz) of $J_{2,3}$ suggests that the dihedral angle of H-2 and H-3 is somewhat greater than 60°, as would be caused by a small torsional displacement along the C-2–C-3 bond. This coupling is of the same order as the vicinal coupling of diequatorial protons in 6-membered-ring systems[8], in contrast to axial–equatorial vicinal-couplings, which are in the range of 3–4 Hz.

Tetra-O-acetyl-L-rhamnose diethyl dithioacetal (**6**). — The coupling data are entirely consistent with the planar, zig-zag arrangement (**6a**) of the carbon atoms in the chain; large values of $J_{2,3}$ and $J_{4,5}$ show that H-2 and H-3, and also H-4 and H-5, are antiparallel, and the value of $J_{3,4}$ (1.8 Hz) indicates that H-3 and H-4 are in a gauche relationship. The other gauche, C-3–C-4 rotamer would have a parallel 1,3-interaction between O-3 and O-5. The value of $J_{1,2}$ (4.7 Hz) is intermediate between that expected for the gauche, H-1–H-2 arrangement and the antiparallel conformer (**6a**), suggesting that there is a substantial contribution from a gauche

conformer, as well as from the antiparallel conformer. This conclusion is not unexpected, because the conformation **6a** introduces a parallel 1,3-interaction between O-3 and one ethylthio group at C-1. The gauche arrangement depicted in **6b** is free of such 1,3-interaction, and can be expected to contribute substantially to the conformational population, even though it does not have the maximum staggering of large groups along C-1–C-2. The second, gauche, H-1–H-2 rotamer would have the parallel 1,3-interaction of O-3 and an ethylthio group, and is, presumably, not favored.

Tetra-O-*acetyl*-D-*lyxose diethyl dithioacetal* (**4**). — The conformation **4a** having the chain of carbon atoms in a planar, zig-zag arrangement is in agreement with the coupling data; $J_{2,3}$ is 7.9 Hz and $J_{3,4}$ is 2.0 Hz. The small value of $J_{3,4}$ may indicate, as for **5** and **6**, that there is some increase in the dihedral angle of the two coupled protons, beyond the nominal 60° in a strictly planar chain. As with **6**, the $J_{1,2}$ coupling does not accord with the antiparallel disposition of H-1 and H-2 as the preponderant conformer; such an arrangement has an unfavorable, parallel 1,3-interaction and, in fact, the gauche conformer (**4b**), having no such interaction, appears to be the favored form.

Tetra-O-*acetyl*-D-*ribose diethyl dithioacetal* (**1**). — The coupling data cannot be reconciled with the planar, zig-zag conformation depicted in **1a**. Although the $J_{1,2}$ and $J_{2,3}$ values (6.2 and 5.7 Hz, respectively) are consistent with major contributions from rotamers having H-1, H-2, and H-3 mutually antiparallel, as required for **1a**, the small (3.6 Hz) $J_{3,4}$ coupling is entirely incompatible with formulation **1a**, and it requires that H-3 and H-4 be gauche-disposed. Conformer **1a** has a parallel 1,3-interaction between O-2 and O-4. Of the two C-3–C-4, gauche rotamers, one has a parallel interaction between O-2 and C-5 and is, presumably, unfavored. The other gauche rotamer (**1b**) appears to be the favored arrangement of the carbon chain; it may be referred to as a "sickle" conformation. The fact that $J_{1,2}$ and $J_{2,3}$ are substantially less than the 8–9 Hz required for antiparallel protons may be attributed to steric destabilization arising from the 1,3-interaction between O-3 and one of the ethylthio groups; this interaction may result in minor distortion of the chain to allow recession of these interacting groups, or it may lead to appreciable contributions by other rotamers.

Tetra-O-*acetyl*-D-*xylose diethyl dithioacetal* (**3**). — As with compound **1**, the coupling data cannot be reconciled with the planar, zig-zag formulation (**3a**), because the $J_{2,3}$ coupling (5.9 Hz) is too large for the protons at C-2 and C-3 to be in the gauche orientation. The large value of $J_{2,3}$ indicates that there is a preponderance of the "sickle" conformation (**3b**) having H-2 and H-3 antiparallel, although one or both of the other C-2–C-3 rotamers (both have a parallel 1,3-interaction of large groups) may make an appreciable contribution to the rotamer population. The value of $J_{1,2}$ is smaller than that expected for the exclusive antiparallel disposition of H-1 and H-2, and indicates some degree of conformational distortion or population of other rotamer states; predictions of the exact dispositions of H-1, H-2, and H-3 are, however, not justified from the coupling data available.

General. — The data of the present investigation and of the previous ones[1,2] demonstrate that the planar, zig-zag conformation resulting from maximum staggering of small–medium–large sets of groups along each carbon–carbon bond is adopted in solution by polysubstituted alkane derivatives (such as **1–6**) as the favored arrangement *provided that this arrangement does not lead to a parallel 1,3-interaction of substituent groups.* The latter interaction is analogous to a 1,3-diaxial interaction of substituents on a 6-membered ring-system. In the case of two interacting oxygen atoms, the nuclei would approach to within 2.50 Å, and generate greater steric destabilization than would be caused by adoption of an alternative rotamer state that would bring the chain of carbon atoms out of the planar, zig-zag arrangement, even though such a conformational change would introduce additional, gauche 1,2-interactions. In a gauche interaction between vicinal oxygen atoms, the nuclei are still 2.95 Å apart, and approach to 2.50 Å in the fully eclipsed conformation only.

Conformational studies by n.m.r. spectroscopy do not furnish precise data on molecular shapes, with exact bond angles, such as are provided by X-ray crystallography or neutron diffraction methods[9]. The n.m.r. method can, however, indicate the shape, in broad terms, of molecules in solution, and it can also furnish information on conformational equilibria[10]. Molecular shapes as determined by crystallographic methods refer to molecules held rigidly (by close-range, intermolecular forces) in the crystal lattice, and do not necessarily correspond to the favored conformation in solution, although such a correspondence is frequently observed. It may be noted that, in two recent examples, crystallographic analysis has demonstrated a "sickle" conformation of a polyhydroxyalkyl chain that would have had a parallel 1,3-interaction of oxygen atoms in a planar, zig-zag arrangement of the carbon chain. The "sickle" conformation is adopted by ribitol and xylitol in the crystalline state[11] and also by the ribitol chain in riboflavine hydrobromide monohydrate[12].

Measurement of favored conformations of acyclic sugar derivatives in solution may furnish data useful in interpretation of the course of ring-forming reactions of sugars and other systems. Related studies on acetylated osotriazoles, acetylated *aldehydo* sugars, and nonacylated aldose dialkyl dithioacetals are to be published.

EXPERIMENTAL

Materials. — The acetylated dithioacetals **1–6** were prepared by standard methods[13], and the purified products had melting points and specific rotations in good agreement with the values recorded in the literature sources noted: **1** (ref. 14), **2** (ref. 15), **3** (ref. 16), **4** (ref. 17), **5** (ref. 18), and **6** (ref. 19).

N.m.r. spectral measurements. — Spectra were measured with a Varian HA-100 n.m.r. spectrometer at ~28°. Samples were ~10%, in chloroform-*d* containing ~5% of tetramethylsilane. The latter was used as an internal standard and to provide a lock signal. Chemical shifts of principal peaks were taken directly from a digital counter that measured the difference in frequency between the lock signal and the signal measured. The shifts are considered accurate to within ±0.1 Hz.

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NEIGHBORING-GROUP PARTICIPATION IN CARBOHYDRATES: THE (METHYLTHIO)CARBONYL NEIGHBORING GROUP

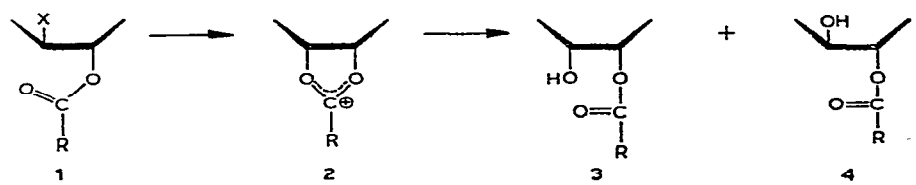
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(Received November 15th, 1968)

ABSTRACT

The use is described of the (methylthio)carbonyl group as a means of preparing carbohydrates having *cis*-oriented functional groups, by way of stable, intermediate, cyclic carbonates. By this route, methyl 2-*O*-*p*-tolylsulfonyl-α-D-arabinopyranoside was prepared from methyl α-D-lyxopyranoside. Methyl α-D-lyxofuranoside was prepared from methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-arabinofuranoside.

RESULTS AND DISCUSSION

The use of neighboring-group participation for effecting a controlled inversion at specific asymmetric centers has been of great utility for the preparation of new sugars. A recent review[1] by Goodman gives a comprehensive survey of the role of participation reactions in sugars. The majority of these reactions proceed *via* a transitory, orthoester ion, such as **2**, which is opened to give either *cis* (**3**) or *trans* (**4**) bifunctional products as the end product.

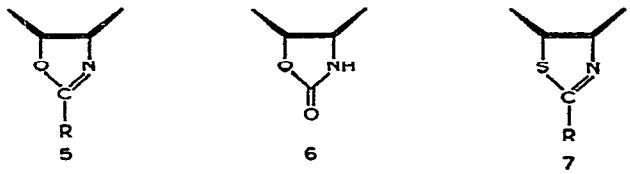


1 2 3 4

The relative proportions of *cis* and *trans* products are dependent on the reaction conditions[2]. A recent report[3] on the synthesis of 9-β-D-lyxofuranosyladenine, corresponding to **3** from a derivative of 9-β-D-xylofuranosyladenine, corresponding to **1**, described the conditions necessary for obtaining the maximum ratio of *cis* to *trans* products. In this example, as in the majority of others involving *O*-acyl participation through an orthoester ion, the ultimate product consisted of a mixture of *cis* and *trans* compounds, their ratio depending on the reagents and starting materials employed. The separation of the pure product can be a tedious operation[3].

More specific are neighboring-group reactions in amino sugar chemistry; they

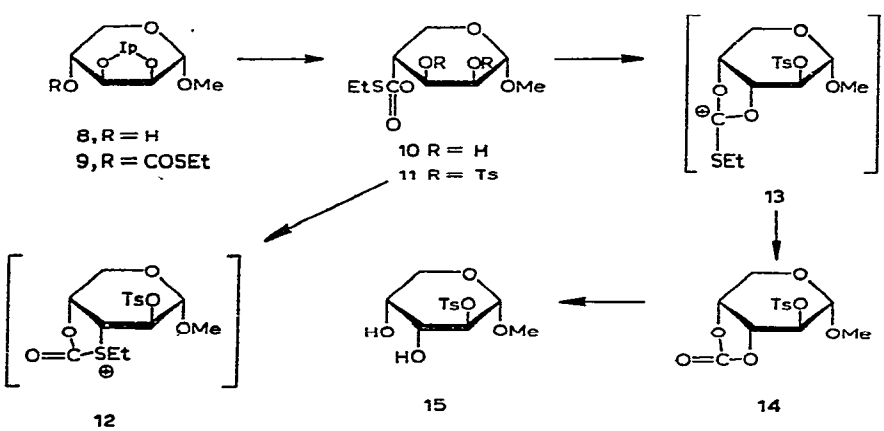
give relatively stable, *uncharged*, cyclic intermediates (such as 5–7), that may[4] or may not[5] be hydrolyzed under the conditions of the reaction.



5 6 7

As far as nitrogen-free carbohydrates are concerned, apparently no example has been reported of the use of a carbonate participating group, although the latter should react in a similar way, to give a cyclic carbonate. Saponification would result in a *cis* product. The use of an *S*-alkyl thiocarbonate, such as **10**, would give the added possibility that participation could occur through sulfur, and thus serve as a convenient method for the introduction of sulfur into the sugar molecule. An investigation of the use of the *S*-ethylthiocarbonate in this reaction is described in this paper.

Treatment of methyl 2,3-*O*-isopropylidene-α-D-lyxopyranoside[6] (**8**) with *S*-ethyl chlorothioformate gave methyl 4-*O*-(ethylthio)carbonyl-2,3-*O*-isopropylidene-α-D-lyxopyranoside (**9**) as a distillable oil. Deacetonation of **9** with aqueous acetic acid gave crystalline methyl 4-*O*-(ethylthio)carbonyl-α-D-lyxopyranoside (**10**). Esterification of **10** with *p*-toluenesulfonyl chloride gave a good yield of crystalline methyl 4-*O*-(ethylthio)carbonyl-2,3-di-*O*-*p*-tolylsulfonyl-α-D-lyxopyranoside (**11**), the starting material desired for the participation reaction.



8, R = H
9, R = COSEt

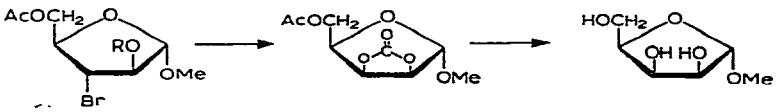
10 R = H
11 R = Ts

12 13 14 15

Treatment of **11** with sodium fluoride in anhydrous *N*,*N*-dimethylformamide for 3 days at 140° gave a semicrystalline solid, from which crystalline methyl 2-*O*-*p*-tolylsulfonyl-α-D-arabinopyranoside 3,4-cyclic carbonate (**14**) was readily isolated. There was no evidence for any products that might have arisen from participation by sulfur; however, the yield of crude product was quite low, so the possibility that participation by sulfur occurred, by way of **12**, to give water-soluble products has not yet been eliminated.

Deacetylation of **14** gave crystalline methyl 2-*O*-*p*-tolylsulfonyl-α-D-arabinopyranoside (**15**), having identical properties (except for the optical rotation, which was equal but opposite) with those reported for methyl 2-*O*-*p*-tolylsulfonyl-α-L-arabinopyranoside[7].

A similar participation-reaction was demonstrated in the furanose series, although the reaction appeared to be slower. Methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-arabinofuranoside[8] (**16**) was acylated with *S*-ethyl chlorothioformate in pyridine, to give methyl 5-*O*-acetyl-3-bromo-3-deoxy-2-*O*-(ethylthio)carbonyl-α-D-arabinofuranoside (**17**) as an analytically pure oil. Treatment of **17** with sodium fluoride in dry *N*,*N*-dimethylformamide for 7 days at 150° gave an oil that contained approximately equal amounts of starting material (**17**) and product (**18**), as estimated by the relative intensities of the carbonyl absorption in the i.r. spectrum. The separation of unreacted **17** from methyl 5-*O*-acetyl-α-D-lyxofuranoside 2,3-cyclic carbonate (**18**)



16 , R = H
17 , R = COSEt

18

19

was accomplished by means of thick-layer chromatography on silica gel. Deacylation of **18** with methanolic sodium methoxide gave methyl α-D-lyxofuranoside (**19**), having physical properties in good agreement with those reported[9]. As in the case of the pyranoside **11**, no products that could have arisen by participation of sulfur were isolated.

The use of the acyclic carbonic ester as a source of anchimeric assistance for the preparation of *cis* derivatives of sugars should be useful, especially in such syntheses as that of lyxofuranosyladenine, where the normal, orthoester-ion intermediate gives mixtures of *cis* and *trans* products that are difficult to separate.

EXPERIMENTAL

General methods. — Melting points are corrected. Thin-layer chromatograms were performed on Silica Gel HF (E. Merck AG, Darmstadt). The solvents used were: *A*, 9:1 benzene–ethyl acetate; *B*, 1:3 cyclohexane–ethyl ether. Spots were detected with iodine vapor. Solutions in organic solvents were dried with anhydrous magnesium sulfate.

Methyl 4-O-(ethylthio)carbonyl-2,3-O-isopropylidene-α-D-lyxopyranoside (**9**). — A solution of 5.0 g (23.3 mmoles) of methyl 2,3-*O*-isopropylidene-α-D-lyxopyranoside[6] (**8**) in 100 ml of dry pyridine was cooled to 0° under nitrogen, and 10.0 ml (105 mmoles) of *S*-ethyl chlorothioformate was added dropwise, with stirring and continued cooling. After the addition was complete, the mixture was stirred for 18 h at room temperature.

The solution was cooled in an ice bath, and the excess of reagent was decom-

posed by the addition of 10 drops of water. The mixture was partitioned between ether (50 ml) and saturated aqueous sodium hydrogen carbonate (50 ml). The ether layer was washed with water, dried, and evaporated to dryness to give 7.38 g of crude product as an oil. Purification by distillation gave 4.7 g (65%) of **9**, b.p. 140°/0.08 torr; λ_{max}^{film} 5.78 (C=O), 8.70 μm (C–O–C).

Anal. Calc. for $C_{12}H_{20}O_6S$: C, 49.4; H, 6.85; S, 11.0. Found: C, 49.7; H, 6.90; S, 11.3.

Methyl 4-O-(ethylthio)carbonyl-α-D-lyxopyranoside (**10**). — A suspension of 4.5 g (15.4 mmoles) of **9** in 70 ml of 60% aqueous acetic acid was heated with stirring for 3 h at 70–75°, by which time, dissolution was complete. The solution was evaporated to dryness *in vacuo*, and the last traces of acetic acid were removed by the addition and evaporation of toluene (10 ml), to leave 3.59 g of crude **10** as a colorless solid, m.p. 112–123°, which was satisfactory for the next step.

An analytical sample of **10**, prepared by recrystallization from benzene, had m.p. 128.5–129.0°; $[\alpha]_D^{22}$ +17° (*c* 1, methanol); λ_{max}^{Nujol} 2.90 (OH) 5.78 (C=O), 8.60 μm (C–O–C).

Anal. Calc. for $C_9H_{16}O_6S$: C, 42.9; H, 6.40; S, 12.7. Found: C, 43.2; H, 6.35; S, 13.0.

Methyl 4-O-(ethylthio)carbonyl-2,3-di-O-p-tolylsulfonyl-α-D-lyxopyranoside (**11**). — A solution of 2.88 g (11.4 mmoles) of **10** in 50 ml of dry pyridine was cooled to 0° under nitrogen, and 10.9 g (57.2 mmoles) of *p*-toluenesulfonyl chloride was added, with stirring and continued cooling. After ~0.5 h at 0°, the mixture was stirred for 18 h at room temperature. The excess of *p*-toluenesulfonyl chloride was decomposed by the addition of a small amount of ice, and the mixture was partitioned between chloroform (30 ml) and water (30 ml). The chloroform layer was washed successively with saturated aqueous sodium hydrogen carbonate, and water, dried, and evaporated to dryness *in vacuo*, to give 6.55 g of crude product as an oil. Trituration of the oil with methanol gave 5.15 g (81%) of crystals, m.p. 91–94°.

An analytical sample was obtained by recrystallization from methanol, and had m.p. 93.5–95.5°; λ_{max}^{Nujol} 5.75 (C=O); 8.31, 8.45 (SO_2), and 8.75 μm (C–O–C).

Anal. Calc. for $C_{23}H_{28}O_{10}S_3$: C, 49.4; H, 5.04; S, 17.2. Found: C, 49.5; H, 5.02; S, 17.3.

Methyl 2-O-p-tolylsulfonyl-α-D-arabinopyranoside 3,4-cyclic carbonate (**14**). — A suspension of 1.9 g of dry sodium fluoride and 2.0 g (3.6 mmoles) of **11** in 120 ml of dry *N,N*-dimethylformamide was heated for 72 h at 145°, with stirring under nitrogen. The mixture was cooled, water (1 ml) was added, and the mixture was stirred for 2–3 h and evaporated to dryness *in vacuo*. The residue was partitioned between ether (30 ml) and water (30 ml). The ether layer was successively washed with saturated aqueous sodium hydrogen carbonate and water, and dried. The solution was evaporated to dryness *in vacuo*, to give 0.77 g (62%) of crude product as a semicrystalline solid which, on trituration with methanol gave 0.25 g of crystals, m.p. 109.5–111.5°. T.l.c. of the mother liquors with solvent *A* showed them mainly to contain **14** (R_F 0.3), together with several trace components.

An analytical sample was obtained by recrystallization from methanol, and had m.p. 113.5–115.0°; λ_{max}^{Nujol} 5.45 (cyclic carbonate C=O) and 8.45 μm (SO_2^-).

Anal. Calc. for $C_{14}H_{16}O_8S$: C, 48.9; H, 4.66; S, 9.30. Found: C, 49.3; H, 4.59; S, 9.58.

Methyl 2-O-p-tolylsulfonyl-α-D-arabinopyranoside (**15**). — A solution of 100 mg (0.29 mmoles) of **14** in 2 ml of 10 mM methanolic sodium methoxide was stirred for 2 h at room temperature and then the base was neutralized with Dowex-50 (H^+) ion-exchange resin. The resin was filtered off, and the filtrate was evaporated to dryness *in vacuo*, to give 80 mg of product as a colorless solid which was partitioned between chloroform and water. The chloroform layer was dried, and evaporated to dryness in *vacuo* to give 75 mg of an oil that crystallized when triturated with ether; it had m.p. 131.5–132.5°; $[\alpha]_D^{24}$ +17° (*c* 1.0, chloroform); λ_{max}^{Nujol} 2.90, 2.98 (OH) 7.30–7.45 μm (OSO_2^-).

Mukherjee and Todd[7] reported m.p. 129–130°, $[\alpha]_D$ −15° (*c* 4.88, chloroform) for methyl 2-*O*-*p*-tolylsulfonyl-α-L-arabinopyranoside.

Methyl 5-O-acetyl-3-bromo-3-deoxy-2-O-(ethylthio)carbonyl-α-D-arabinofuranoside (**17**). — A solution of 2.0 g (7.4 mmoles) of methyl 5-*O*-acetyl-3-bromo-3-deoxy-α-D-arabinofuranoside[8] (**16**) in 25 ml of dry pyridine was cooled to 0° under nitrogen, and 2.4 ml (25 mmoles) of *S*-ethyl chlorothioformate was added dropwise, with stirring. After the addition was complete, the reaction was kept overnight at room temperature.

The excess of acid chloride was decomposed by adding ~1 g of ice and stirring for 1 h. The mixture was partitioned between ether (25 ml) and water (25 ml). The ether layer was successively washed with saturated, aqueous sodium hydrogen carbonate and water, dried, and evaporated to dryness *in vacuo*, to give 2.9 g of product as an oil. A small amount of inorganic material was removed by treating the product with methanol, and filtering to remove an insoluble precipitate. The filtrate was evaporated to dryness *in vacuo*, and dried at 0.1 torr for 24 h, to give 2.85 g of material having $[\alpha]_D^{21}$ +44° (*c* 1.0, chloroform); λ_{max}^{film} 5.75, 5.88 (C=O); 8.15 (acetate C–O–C), and 8.85 μm (thiocarbonate C–O–C).

Anal. Calc. for $C_{11}H_{17}BrO_6S$: C, 37.0; H, 4.80; S, 8.98; Br, 22.4. Found: C, 37.3; H, 4.80; S, 8.79; Br, 22.3.

Methyl 5-O-acetyl-α-D-lyxofuranoside 2,3-cyclic carbonate (**18**). — A mixture of 2.0 g (5.6 mmoles) of **17** and 2.4 g of sodium fluoride in 200 ml of dry *N*,*N*-dimethylformamide was heated for 7 days at 150°, with stirring under nitrogen. The mixture was cooled to room temperature, 1 ml of water was added, and the mixture was stirred for 4 h and then evaporated to dryness *in vacuo*.

The residue was partitioned between ether and water. The ether layer was successively washed with saturated, aqueous sodium hydrogen carbonate and water, dried, and evaporated to dryness *in vacuo*, to give 1.24 g of an oily residue. T.l.c. (solvent *B*) showed major spots at R_F 0.22 (assigned to the product, **24**) and R_F 0.82 assigned to **17**, together with trace components having R_F values of 0.65 and 0.10.

The pure product was isolated by thick-layer chromatography with solvent *B*,

to give 0.3 g of **18** as an oil, λ_{max}^{film} 5.50, 5.71 (C=O) 8.10 (acetate C–O–C), and 8.60 μm (cyclic carbonate C–O–C).

Anal. Calc. for $C_9H_{12}O_7$: C, 46.6; H, 5.21. Found: C, 46.9; H, 5.43.

Methyl α-D-lyxofuranoside (**19**). — A solution of 100 mg of **18** in 4 ml of 10 mM methanolic sodium methoxide was stirred under nitrogen for 18 h at room temperature neutralized to pH 7 with Dowex-50 (H^+) ion-exchange resin, and filtered through a Celite pad. The filtrate was evaporated to dryness *in vacuo*, to give 71 mg of product as an oil. Crystallization was effected by trituration with ether. Recrystallization from ethyl acetate gave methyl α-D-lyxofuranoside (**19**) as colorless crystals, m.p. 97.5–98.5°; $[\alpha]_D^{22}$ +128° (*c* 1, methanol); lit.[9] m.p. 96.5–97°; $[\alpha]_D$ +128° (water).

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NEIGHBOURING-GROUP EFFECTS IN THE CHEMISTRY OF 3,4-ANHYDRO-D-ALTRITOL

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ABSTRACT

3,4-Anhydro-D-altritol has been prepared by the partial, acidic hydrolysis of its 1,2:5,6-di-*O*-isopropylidene derivative. When treated with 2.5N sodium hydroxide, 3,4-anhydro-D-altritol yields 1,4-anhydro-D-altritol and 1,5-anhydro-L-glucitol as the sole products; 2,3-anhydro-D-iditol is postulated as an intermediate. 2,3-Anhydro-1,6-di-*O*-triphenylmethyl-D-iditol, prepared by epoxide migration of 3,4-anhydro-1,6-di-*O*-triphenylmethyl-D-altritol, yields 1,4-anhydro-D-altritol and 1,5-anhydro-L-glucitol on acidic hydrolysis. Anhydrohexitols are minor products in the hydrolysis of 3,4-anhydro-D-altritol by 0.1N sulphuric acid. Neighbouring acetoxyl-group participation is evident during the acidic hydrolysis of 1,2,5,6-tetra-*O*-acetyl-3,4-anhydro-D-altritol.

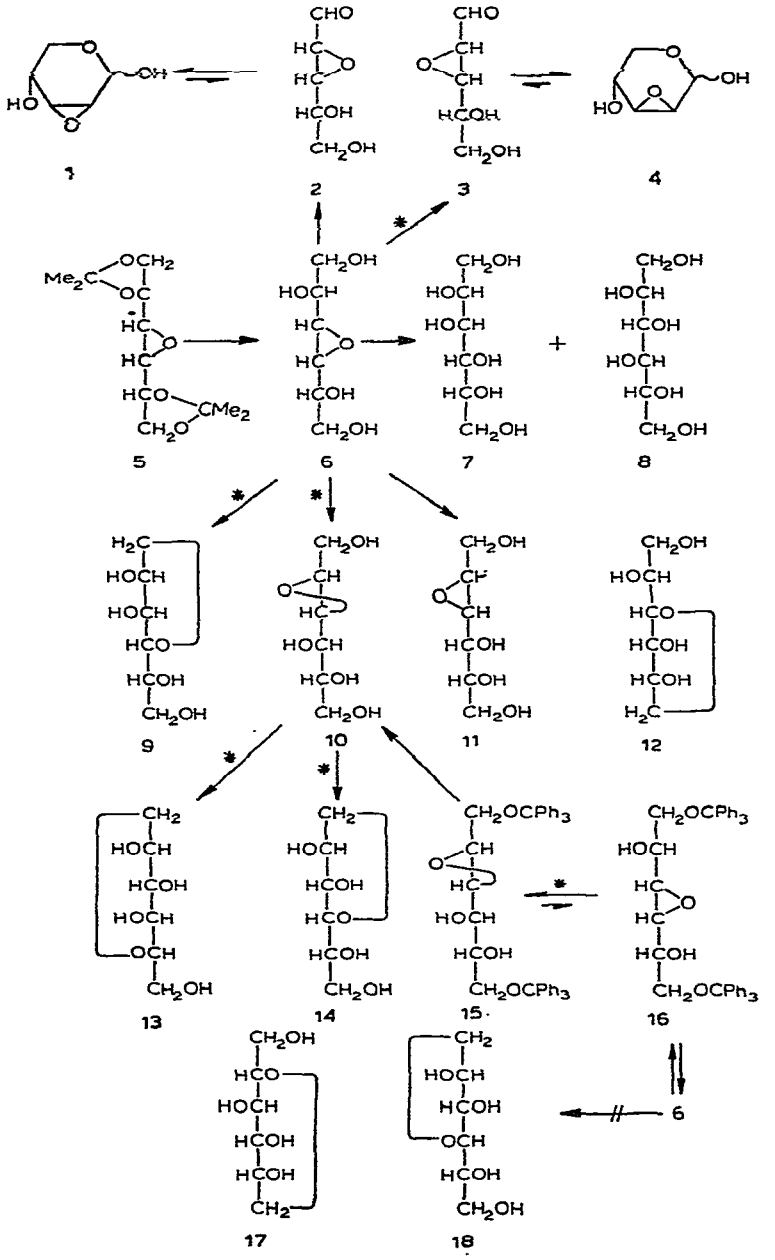
INTRODUCTION

In 1950, Bladon and Owen[1] described the preparation of 3,4-anhydro-1,2:5,6-di-*O*-isopropylidene-D-altritol (**5**) and its behaviour towards acidic and basic reagents. Hydrolysis with aqueous sulphuric acid yielded D-mannitol (**7**) and D-iditol (**8**). Since cleavage of the epoxide ring in compound **5** by sodium methoxide was very slow, probably for steric reasons, it seemed likely that, in the acidic hydrolysis, 3,4-anhydro-D-altritol (**6**) would be an intermediate, prior hydrolysis of the acetal residues having taken place. As part of our study of epoxide migrations[2] and related reactions[3], we wished to prepare the anhydroaltritol **6** and to examine its behaviour towards hydrolysis. 3,4-Anhydro-2,5-*O*-methylene-1,6-di-*O*-triphenylmethyl-D-altritol[4] was unsuitable for our purpose because of the stability of the methylene bridge.

RESULTS AND DISCUSSION

When the acetal **5** was hydrolysed with 80% acetic acid for 10 min at 100°, 3,4-anhydro-D-altritol (**6**) could be isolated in 89% yield after silica gel chromatography. The epoxide **6** reduced 2.1 mol. of sodium periodate in 48 h, the first mol. requiring 15 min. The early products of periodate oxidation were shown by paper chromatography to be 2,3-anhydro-D-ribose [(**2**) and (**1**)] and 2,3-anhydro-D-lyxose

[(3) and (4)]. The longer time required for the reduction of the second mol. of periodate is due to the preferential existence of the ring forms **1** and **4**.



*In these transformations the Fischer projection formulae have been turned through 180° in order to conform to rules of nomenclature.

When the anhydroaltritol **6** was treated with 2.5N sodium hydroxide for 1.5 h at 100°, only two products were detected by paper chromatography. They were

easily separated by chromatography on a basic ion-exchange resin[5], and shown to be 1,4-anhydro-D-altritol[6] (**14**; 53%) and 1,5-anhydro-L-glucitol (**13**; 27%) by comparison with authentic compounds. No free hexitol was detected. In order to account for the configurations of the products, the most likely reaction sequence involves formation of 2,3-anhydro-D-iditol (**10**) by epoxide migration[7], followed by intramolecular attack by the C-6 alkoxy-anion on C-3 or C-2. There are several precedents for the formation of a 5-membered ring in this way[8-10]. Study of a Dreiding model of compound **10** shows that ring closure to give a 6-membered ring should also readily occur, since the all-equatorial conformation of the anhydroglucitol **13** can be achieved in the transition state. The preference for the five-membered ring is to be expected[11], since ring-closure is irreversible.

Neither of the products of alkaline hydrolysis of epoxide **6** was derived directly by intramolecular attack by a terminal alkoxy-anion. In ring-opening reactions of cyclohexene oxides, the nucleophile approaches from an axial direction[12], and this is the basis of the Fürst–Plattner rule. A geometrically similar approach should occur in the case of vicinal epoxides which are not fused to another ring, and also in the case of intramolecular ring-opening. A study of Dreiding models shows that it is more difficult to reach a satisfactory position for such intramolecular attack in the anhydroaltritol **6** than in the anhydroiditol **10**. This is borne out by some earlier work[8,9,13-16]. For example, alkaline hydrolysis of 1,2-epoxy-4-butanol yielded[13] only 1,2,4-butanetriol. Even in the cases of 5,6-anhydro-1,2-*O*-isopropylidene-α-D-glucofuranose[14,15] and -β-L-idofuranose[15,16], where the C-3 hydroxyl group is favourably orientated for intramolecular attack, 3,6- and 3,5-anhydrides are relatively minor products.

In principle, epoxide migration in the anhydroaltritol **6** can take place in either direction to give either the anhydroiditol **10** or 2,3-anhydro-D-mannitol (**11**). The former should be favoured thermodynamically, since it is a *trans*- epoxide, and kinetically, since the C-5 oxygen atom has an *erythro* relationship to C-4 in the anhydroaltritol **6**. The intramolecular nucleophilic attack by the C-6 oxygen atom in the anhydroiditol **10** must occur very rapidly, because no other products were detected, *e.g.*, those which would be expected to arise from the terminal epoxide. We decided to study epoxide migration in a derivative of epoxide **6** in which the primary hydroxyl groups were protected in order to prevent their participation in intramolecular reactions.

Compound **6** was converted into its di(triphenylmethyl)ether **16**, which was treated with sodium methoxide in methanol to give 2,3-anhydro-1,6-di-*O*-triphenylmethyl-D-iditol (**15**) (84% yield). Whereas the anhydroaltritol **16** could be converted back into the epoxide **6** by brief treatment with 80% acetic acid, similar treatment of the anhydroiditol **15** gave only 1,4-anhydro-D-altritol (**14**) and 1,5-anhydro-L-glucitol (**13**) in approximately the same ratio as in the alkaline treatment of epoxide **6**. No anhydroiditol **10** could be detected by paper chromatography. It is known that vicinal epoxides bearing a suitably situated hydroxyl group are susceptible to intramolecular scission under acidic[3,17-21] as well as alkaline conditions. In particular,

2,3-anhydrohexoses have been shown to be readily transformed into 3,6-anhydrohexoses by acid[3], and the products obtained by acidic hydrolysis of epoxide **15** are a clear indication that it has the 2,3-anhydro-D-*ido* structure shown. Although it is possible to envisage 4-membered oxides (oxetanes) as intermediates in the above transformations, we do not consider this likely, either in these experiments, or in those to be described below.

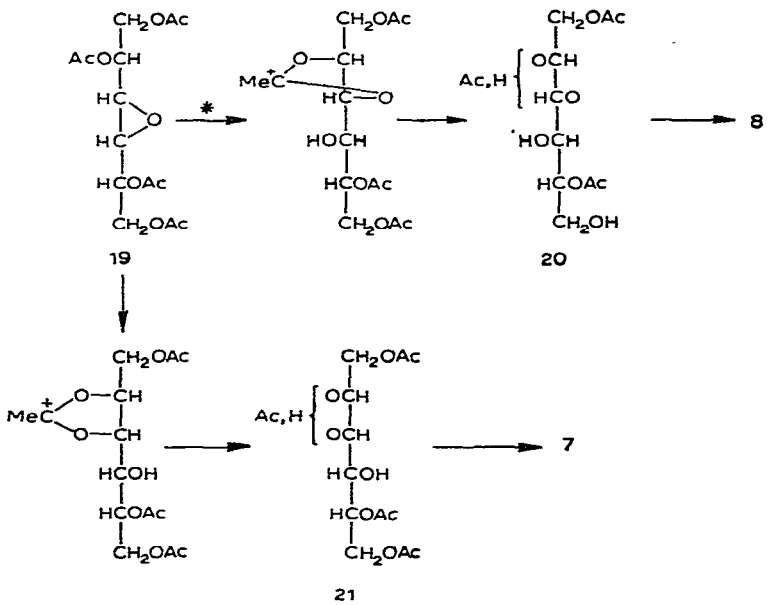
In the light of these results, we re-investigated the hydrolysis[1] of the diacetal **5** by dilute sulphuric acid. Paper chromatography showed that the main product was a mixture of hexitols, and that smaller amounts of anhydrohexitols were also present. The products were separated, by chromatography and by crystallisation, to give D-iditol (**8**; 45%, as the hexa-acetate), D-mannitol (**7**; 33%), 1,4-anhydro-D-altritol (**14**; 4%), 1,5-anhydro-L-glucitol (**13**; 3%), 3,6-anhydro-D-altritol (**12**; 3%), and 1,4-anhydro-D-mannitol (**9**; 2%). The isolation of D-iditol (**8**) and D-mannitol (**7**) as the major products is in complete agreement with the results of Bladon and Owen[1]. These products clearly arise by simple, acid hydrolysis of the 3,4-epoxide ring. The mixture of anhydrohexitols was more complex than that arising from the alkaline reaction. Two of the compounds, **13** and **14**, were the major products from the treatment of the anhydroaltritol **6** with alkali, and it would appear that some epoxide migration to the anhydroiditol **10** has occurred under acidic conditions. 3,6-Anhydro-D-altritol (**12**) would then arise from the 2,3-anhydromannitol **11**, the other possible epoxide-migration product, and 1,4-anhydro-D-mannitol (**9**) by intramolecular ring-opening in the 3,4-anhydroaltritol **6** itself. There has been no previous indication of epoxide migration under acidic conditions in the chemistry of epoxides of cyclic sugars[3] or of inositols[7]. It is possible, however, that such reactions might be more favoured in an acyclic case where free rotation may assist in the attainment of the required transition state. Hudson and Barker[22] have recently invoked vicinal epoxide formation in order to explain the multiplicity of anhydropentitols resulting from the acid-catalysed dehydration of D-arabinitol which, due to steric factors, reacts more slowly than ribitol or xylitol.

It is interesting that, among the anhydrohexitol products arising from treatment of epoxide **6** with acid, no 2,6-anhydro-D-glucitol (**17**) or 1,4-anhydro-D-iditol (**18**) were isolated. The anhydroglucitol **17** is the six-membered ring product expected to arise from the 2,3-anhydromannitol **11**. Its possible presence cannot be excluded, because an authentic sample was not available. The 1,4-anhydroiditol **18** might be expected to be formed, together with the 1,4-anhydromannitol **9**, by intramolecular attack of a terminal hydroxyl group on the 3,4-epoxide ring of compound **6**. It was shown not to be a product by paper-chromatographic comparison of hydrolysis mixtures with the authentic compound. When the two cyclisation processes (**6**→**9**; **6**→**18**) are studied by means of Dreiding models, the one leading to compound **9** is more favourable than that leading to compound **18**. In the latter case, hindrance to attack at C-4, due to the C-5 hydroxyl group, occurs in the extended conformation of the carbon chain.

We considered the possibility that some of the anhydrohexitol products from

the acidic hydrolysis of epoxide **6** might arise through the formation of sulphuric esters (*cf.* ref. 23). It was found, however, that the use of dilute perchloric acid, whose anion is a very weak nucleophile, or additions of sulphate ion had no effect on the product distribution. It is concluded that ester intermediates are not involved.

It was of some interest to examine the acid hydrolysis of the tetra-acetate **19** of the 3,4-anhydroaltritol **6**. It would be expected that hydrolysis would occur much more easily than in the parent epoxide **6**, because of neighbouring-group participation by a vicinal acetoxy-group[24-26]. In cyclic systems, there is a stereochemical requirement that the neighbouring acetoxy-group must be *trans* to the epoxide ring. In the case of compound **19**, either the C-5 acetoxy-group or that at C-2 should be capable of participation, with the formation of tetraacetates (**20**, **21**) of D-iditol and D-mannitol, respectively. It would be expected that the C-5 acetoxy-group in compound **19**, having the *erythro*-relationship to C-4, would participate more readily than the C-2 acetoxy group. These expectations were borne out when it was found that hydrolysis of the acetate **19** with 80% acetic acid at 100° was complete in 15 min, conditions which hardly affect compound **6**. The crude product, presumably a mixture of compounds **20** and **21**, was deacetylated and examined by paper chromatography. Iditol (**8**) and mannitol (**7**) were the sole products, and were characterised as D-iditol hexaacetate (80%) and D-mannitol (15%). In the acidic hydrolysis of epoxide **6**, the iditol:mannitol ratio was 45:33.



*In this transformation the formula has been turned through 180°.

There has recently been renewed interest in vicinal epoxides derived from hexitols[27,28].

EXPERIMENTAL

General methods. — Evaporations were carried out under diminished pressure with a bath temperature below 40°. Infrared spectra were measured for potassium bromide discs. Melting points are uncorrected. Comparison of materials with authentic substances was made, unless stated otherwise, by mixed m.p. determination, infrared spectra, and paper chromatography. "80% Acetic acid" refers to acetic acid–water (4:1, v/v).

Chromatographic methods. — Adsorption chromatography was carried out with silica gel (Hopkin and Williams). Triphenylmethyl ethers were chromatographed on silica gel which had been neutralised with ammonia and reactivated[25]. Cellulose columns were made from Whatman cellulose powder (Standard Grade; W. and R. Balston Ltd). Thin-layer chromatography (t.l.c.) was carried out with Kieselgel G (Merck) as adsorbent; compounds were detected with anisaldehyde–sulphuric acid[29]. Paper chromatography was by the descending technique, using Whatman No. 1 paper, except in the case of hexitols, when No. 4 paper was used. Solvent *A* was butanone, saturated with water, the paper being pre-equilibrated in the solvent vapour before irrigation; solvent *B* was butyl alcohol–pyridine–water (3:1:1 by vol). Hexitols and reducing sugars were detected by alkaline silver nitrate[30], vicinal glycols by periodate and Schiff's reagent[31], and vicinal epoxides by sodium iodide and methyl red[25]. The latter spray was also used on t.l.c.

3,4-Anhydro-D-altritol (**6**). — The acetal[1] **5** (6 g) was heated for 10 min at 100° with 80% acetic acid (10 ml). Paper chromatography in solvent *A* showed that the major product gave a purple colour rapidly with periodate and Schiff's reagent, and a yellow colour with sodium iodide–methyl red. The solution was evaporated to dryness, the residual syrup dissolved in chloroform (50 ml), and the solution chromatographed on a column of silica gel (65 g). Chloroform–ethanol (3:2) eluted compound **6** which was obtained as a syrup (3.5 g, 89%), $[\alpha]_D$ +7.8° (*c* 2.12, water) (Found: C, 43.5; H, 7.6. $C_6H_{12}O_5$ calc.: C, 43.9; H, 7.4%).

The compound reduced periodate[32] as follows: 0.98 mol. (15 min), 1.4 mol. (45 min), 1.6 mol. (6 h), 2.1 mol. (2 and 4 days).

3,4-Anhydro-D-altritol (**6**; 30 mg) was treated with a solution of sodium periodate (40 mg) in water (1 ml). After 1 h, periodate and iodate were removed by precipitation with barium chloride. Paper chromatography of the product (solvent *A*) showed the presence of two compounds, each reacting with alkaline silver nitrate and with sodium iodide–methyl red. They had the same R_F value as 2,3-anhydro-L-ribose (0.50) and 2,3-anhydro-L-lyxose (0.57), chromatographic samples of which were prepared by brief, acid hydrolysis[3] (0.1 N sulphuric acid for 20 min at 100°) of the corresponding methyl β-L-glycopyranosides[2,33].

Alkaline hydrolysis of 3,4-anhydro-D-altritol (**6**). — Compound **6** (3.0 g) was heated for 1.5 h at 100° with 2.5N sodium hydroxide (10 ml). The solution was passed through a column of Dowex-50 (NH_4^+) resin, and the combined eluate and washings evaporated to a syrup. Paper-chromatographic examination of the

TABLE I

PAPER CHROMATOGRAPHY OF HEXITOL DERIVATIVES

Compound	R_F *Values*		*Colour with periodate-Schiff's reagent*[31]	
	Solvent A	*Solvent* B	*5 min*	*2 h*
D-Mannitol (7)	0.015	0.115	Purple	Purple
D-Iditol (8)	0.015	0.10	Purple	Purple
3,4-Anhydro-D-altritol (6)	0.17	0.43	Purple	Purple
1,4-Anhydro-D-altritol (14)	0.16	0.42	Purple[a]	Green
3,6-Anhydro-D-altritol (12)	0.07		Yellow[a]	Green[a]
1,5-Anhydro-L-glucitol (13)	0.06	0.30	—	Blue
1,4-Anhydro-D-iditol (18)[b]	0.10		Purple[a]	Green
1,4-Anhydro-D-mannitol (9)	0.12		Yellow[a]	Green[a]

[a]The precise colours are somewhat dependent on the amount of anhydrohexitol present. [b]A chromatographic sample of the L-isomer was prepared by borohydride reduction of 3,6-anhydro-L-idose[8].

product in solvent *A* showed two compounds reacting with periodate and Schiff's reagent; these corresponded to compounds **13** and **14** (see Table I). Alkaline silver nitrate gave no positive reaction on the paper, showing the absence of hexitols.

The syrup (3.0 g) was dissolved in water (5 ml) and chromatographed on a column of Dowex-1 x2 (HO^-) resin (400 ml) by eluting with water[5]. Fractions (40 ml) were collected. Fractions 25–50 yielded a chromatographically pure syrup (0.8 g, 27%) which crystallised on scratching. Recrystallisation from ethanol yielded 1,5-anhydro-L-glucitol (**13**; 0.7 g), m.p. 138°, $[\alpha]_D$ −40.7° (*c* 1.23, water). Its chromatographic properties and infrared spectrum were indistinguishable from those of an authentic sample of 1,5-anhydro-D-glucitol; lit.[34], m.p. 136–138°, $[\alpha]_D$ +41° (water). Fractions 100–140 yielded a pure syrup (1.6 g, 53%) which crystallised on scratching. Recrystallised from ethanol, the product had m.p. 107–109°, $[\alpha]_D$ +12.8° (*c* 1.29, water) and was indistinguishable from an authentic sample of 1,4-anhydro-D-altritol (**14**) kindly given to us by Dr. Robert Barker; lit.[6], m.p. 106–107.5°, $[\alpha]_D$ +13.9° (water).

3,4-Anhydro-1,6-di-O-triphenylmethyl-D-altritol (**16**). — Compound **6** (1.5 g) was treated with triphenylmethyl chloride (5.1 g) in pyridine (75 ml) for 60 h at room temperature. Isolation of the product, using chloroform, yielded a solid foam (5.1 g) which was shown by t.l.c. (benzene–ether, 1:1) to contain a di-*O*-triphenylmethyl-anhydrohexitol as a major component. The crude product (5 g) was dissolved in benzene (10 ml) and chromatographed on neutral silica gel (130 g). Benzene–ether (97:3) eluted triphenylmethanol (0.52 g). Benzene–ether (1:1) eluted a chromatographically homogeneous fraction (by t.l.c.) which, on evaporation, yielded compound **16** as a solid foam, m.p. 75–80°, $[\alpha]_D$ +3.0° (*c* 10.4, chloroform) (Found: C, 80.7; H, 5.9. $C_{44}H_{40}O_5$ calc.: C, 81.4; H, 6.2%).

Hydrolysis of ether **16** *with acetic acid.* — The ether **16** (0.2 g) in 80% acetic acid (1 ml) was heated for 10 min at 100°. Paper chromatography in solvent *A* showed that the product was the anhydroaltritol **6**.

2,3-Anhydro-1,6-di-O-triphenylmethyl-D-iditol (**15**). — The anhydroaltritol **16** (2.5 g) was treated with 0.2N sodium methoxide in methanol (25 ml) for 18 h at room temperature and then for 1 h under reflux. The products were examined by t.l.c. using Kieselgel G which had been spread as a slurry with 2% boric acid[35]. The anhydroaltritol had been completely converted into another di-*O*-triphenylmethyl derivative of an anhydrohexitol having a higher R_F value. Water (30 ml) was added to the above methanolic solution, and the product was isolated by means of chloroform. The resulting syrup (2.3 g) crystallised from aqueous methanol to give the anhydroiditol **15** (2.1 g, 84%), m.p. 85°, $[\alpha]_D$ +10.0° (*c* 10.1, chloroform) (Found: C, 81.6; H, 6.1. $C_{44}H_{40}O_5$ calc.: C, 81.4; H, 6.2%).

Hydrolysis of ether **15** *with acetic acid.* — The ether **15** (1.04 g) was heated with 80% acetic acid (15 ml) at 100°; samples were withdrawn for paper chromatography after 1, 2, 4, 6, 8, 10, and 20 min. Chromatography in solvents *A* and *B* (detection with periodate–Schiff's reagent and with the sodium iodide–methyl red spray, respectively) showed that detritylation was complete within 4 min, and that the products were constant after that time. No compound having the chromatographic properties expected for epoxide **10** was detected at any stage.

After 20 min, the solution was cooled, filtered from triphenylmethanol, and evaporated to a syrup (0.4 g). The syrup was dissolved in water (2 ml) and chromatographed on a column of Dowex-1 x2 (HO^-) resin (50 ml) by eluting with water and collecting 20-ml fractions. Fractions 2–6 were evaporated, and the product was recrystallised from ethanol to yield 1,5-anhydro-L-glucitol (**13**) (57 mg, 22%), m.p. 138–140°, $[\alpha]_D$ −43.1° (*c* 1.0, water), identical with the sample prepared above. Fractions 8–14 were evaporated, and recrystallisation of the product from ethanol yielded 1,4-anhydro-D-altritol (**14**) (128 mg, 49%), m.p. 107°, $[\alpha]_D$ +13.2° (*c* 2.0, water), identical with an authentic sample (see above).

Acid hydrolysis of 3,4-anhydro-1,2:5,6-di-O-isopropylidene-D-altritol (**5**). — The diacetal **5** (3.0 g) was heated with 0.1N sulphuric acid (10 ml) for 4 h at 100°, and the acid was then neutralised with barium carbonate. After filtration, the solution was evaporated to leave a syrup (1.9 g). Chromatography in solvent *A* showed that the main product was hexitol, together with small amounts of compounds corresponding to 1,5-anhydroglucitol, 3,6-anhydroaltritol, 1,4-anhydromannitol, and 1,4-anhydroaltritol (see Table I). 1,4-Anhydroiditol was not detected.

A portion of the syrup (1 g) was dissolved in water (3 ml), placed on a column of cellulose powder (4 × 50 cm) in acetone–water (9:1), and eluted with the same solvent mixture. Fractions were collected, and examined in solvent *A*. The results are shown in Table II.

Fractions 1, 2, and 5 were evaporated to a syrup (62 mg) which was then chromatographed on a column (1.5 × 38 cm) of Dowex-1 x2 (HO^-) resin. Water eluted first 1,5-anhydro-L-glucitol (**13**) (17 mg, 2%), which was recrystallised from ethanol; m.p. 137°, $[\alpha]_D$ −43.0° (*c* 0.9, water). It was indistinguishable from a sample prepared above.

Water next eluted syrupy 3,6-anhydro-D-altritol (**12**; 10 mg) which was com-

bined with fractions 3 and 4 from the cellulose column (total 23 mg, 3%). The syrup was treated overnight with acetone (10 ml) containing conc. sulphuric acid (0.1 ml). Isolation with chloroform yielded a syrup (10 mg) which crystallised. 3,6-Anhydro-1,2:4,5-di-*O*-isopropylidene-D-altritol had m.p. 45°, undepressed when mixed with an authentic sample, m.p. 48–52°. Its infrared spectrum was indistinguishable from that of an authentic sample[3, 23].

TABLE II

FRACTIONATION OF PRODUCTS OF ACID HYDROLYSIS OF COMPOUND **5**.

Fraction No.	*Vol. of fraction (ml)*	*Content of fraction*
1	500	1,4-Anhydro-D-altritol (**14**), 1,4-anhydro-D-mannitol (**9**)
2	50	**14**, **9**, 3,6-Anhydro-D-altritol (**12**)
3	75	**12**
4	75	**12**
5	75	**12**, 1,5-Anhydro-L-glucitol (**13**)
6	25	**13**
7	125	Hexitol
8	975	Hexitol

Water next eluted 1,4-anhydro-D-mannitol (**9**) (12 mg, 2%), which crystallised from ethanol; m.p. 140–142°, indistinguishable from an authentic sample[3,6] prepared from methyl 3,6-anhydro-α-D-mannopyranoside[37] by acid hydrolysis and borohydride reduction.

Finally, water eluted 1,4-anhydro-D-altritol (**14**) (30 mg, 4%). Recrystallised from ethanol, it had m.p. 105–106°, $[\alpha]_D$ +14.2° (*c* 1.0, water), and was indistinguishable from an authentic sample.

Fractions 7 and 8 from the cellulose column were evaporated to a syrup (805 mg), which was dissolved in hot ethanol (5 ml). On cooling, D-mannitol (**7**) (320 mg, 33%) crystallised, m.p. 160–162°, indistinguishable from an authentic sample. Paper chromatography of the mother liquors in solvent *B* showed the presence of iditol with only a little mannitol. The liquors were evaporated to a syrup (450 mg) which was acetylated with acetic anhydride (5 ml) in pyridine (30 ml) overnight. Isolation by means of chloroform yielded D-iditol hexaacetate (809 mg, 45%), m.p. 122–123°, indistinguishable from an authentic sample[3].

Small samples of epoxide **5** were treated severally with 0.1N sulphuric acid, 0.1N sulphuric acid plus sodium sulphate (2 mol.), and 0.1N perchloric acid for 4 h at 100°. The products in each case were examined by paper chromatography in solvent *A* and were indistinguishable.

*1,2,5,6-Tetra-*O*-acetyl-3,4-anhydro-*D*-altritol* (**19**). — Compound **6** (0.5 g) was acetylated with acetic anhydride (3 ml) in pyridine (15 ml) overnight at room temperature. Isolation by means of chloroform yielded a syrup (0.9 g, 87%) (which gave a positive reaction with the sodium iodide–methyl red spray). The acetate, b.p.

140°/10^{-3} mm, had $[\alpha]_D$ +4.4° (*c* 10.3, chloroform) (Found: C, 50.2; H, 5.7. $C_{14}H_{20}O_9$ calc.: 50.6; H, 6.1%).

Hydrolysis of acetate **19** *with acetic acid.* — Acetate **19** (0.5 g) was heated with 80% acetic acid (3 ml) at 100°. Examination of the reaction mixture by t.l.c. (benzene–ether, 1:1) showed that no epoxide remained after 15 min. The solution was evaporated, and the syrupy product was deacetylated with methanolic sodium methoxide. Paper chromatography in solvent *B* showed that the product contained mainly iditol, with a small proportion of mannitol. The hexitol mixture (0.27 g) was acetylated with acetic anhydride (3 ml) in pyridine (10 ml) overnight at room temperature. A chloroform isolation procedure yielded D-iditol hexaacetate (443 mg, 80%) m.p. 122–4° (from ethanol), indistinguishable from an authentic sample[3]. The mother liquors were deacetylated, with sodium methoxide in methanol, to yield D-mannitol (7) (50 mg, 15%), m.p. 165° (from ethanol), identical with an authentic sample.

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Note

Some applications of the Wittig reaction in the synthesis of unsaturated carbohydrates

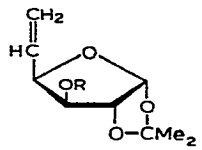
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Our studies on the addition of pseudohalogens to unsaturated carbohydrate derivatives[1] have required suitable olefinic substrates. One such compound, which we[1] and others[2] have found useful in studies of addition reactions, is 5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**1**). This sugar has been prepared by an elimination reaction from 1,2-*O*-isopropylidene-5,6-di-*O*-*p*-tolylsulfonyl-α-D-glucofuranose[3] and by treatment of a 5,6-thionocarbonate with trimethyl phosphite[4]. In an endeavor to find a more convenient synthesis of **1**, we have investigated the reaction of methylenetriphenylphosphorane with 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose[5,6] and its 3-acetate.

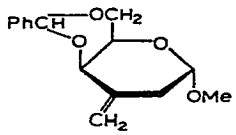
It has been shown[6] that oxidation of 1,2-*O*-isopropylidene-α-D-glucofuranose with sodium periodate, or lead tetraacetate in benzene, does not yield the free aldehyde, 1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose, but a dimer (the 5,5′:3′5-cyclic acetal[5]) and 1,2-*O*-isopropylidene-3,5-*O*-methylene-(5-hydroxy-α-D-xylofuranose). Nevertheless, the glycol-cleavage products do react with Grignard reagents[6-8]. Our attempts to perform a Wittig reaction with the reagent generated from methyltriphenylphosphonium bromide were, however, unsuccessful. The reaction was attempted by using the alkoxide procedure[9] for the preparation of alkylidene phosphoranes, and with the pregenerated phosphorane[10] dissolved in ether. In both cases, only dark, intractable materials were isolated.

A Wittig reaction did occur between methylenetriphenylphosphorane and 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose. This free aldehyde was prepared by oxidation of 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose[11] with sodium periodate; the syrupy product had i.r. and n.m.r. spectra consistent with the expected structure. The Wittig reaction was performed in ether, and involved preparing the ylid in liquid ammonia, with sodium amide as the base, and then replacing the ammonia by ether[10]. As the reaction proceeded, a dark, resinous precipitate was formed. Two compounds were isolated in low yields from the ether solution; these were 3-*O*-acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**2**) and its deacetylated derivative (**1**). The low yields, which make the method unsuitable as a preparative route, are probably attributable to partial deacetylation of the starting aldehyde.

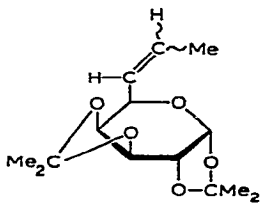
A higher yield of an unsaturated carbohydrate was obtained by the reaction of the same Wittig reagent with methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*threo*-hexopyranosid-3-ulose[12,13]. The product, methyl 4,6-*O*-benzylidene-2,3-dideoxy-3-*C*-methylene-α-D-*threo*-hexopyranoside (**3**), was isolated crystalline by column chromatography. The preparation of branched-chain sugars by application of a modified Wittig reaction to ketoses has been reported[14]. In that work, phosphonoacetic acid trimethyl ester, in the presence of potassium *tert*-butoxide, was used for introducing a (methoxycarbonyl)methylene group in the place of the oxo function.



1 R = H
2 R = Ac



3



4

Finally, we report the reaction of ethylidenetriphenylphosphorane with 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose[15,16] to give 6,7,8-trideoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-oct-6-enose (**4**) in good yield. The n.m.r. spectrum of **4** showed the presence of a three-proton multiplet at τ 4.0–4.7 attributable to H-1 and the two vinyl protons. The pattern was complex, and no information about the configuration of the olefin could be obtained. Compound **4** is of particular interest, as it may be suitable for conversion, by way of a pseudohalogen addition reaction, into the carbohydrate moiety in the antibiotic lincomycin[16,17].

EXPERIMENTAL

General methods. — Melting points were determined using a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 automatic polarimeter at 21 ±2°. I.r. spectra were recorded with a Beckman-IR5A spectrophotometer. N.m.r. spectra were recorded at 60 MHz in chloroform-*d* with tetramethylsilane as the internal standard. Gas–liquid chromatography (g.l.c.) (F and M chromatograph, Model 402) was performed at 185° with a column (115 × 0.4 cm) of 1% (w/w) Apiezon L supported on 50–60 mesh, acid-washed, DMCS-treated Chromosorb W, with helium as the carrier gas. Thin-layer chromatography (t.l.c.) was performed with Silica Gel G as the adsorbent. The developed plates

were air-dried, sprayed with 5% ethanolic sulfuric acid, and heated at about 150°. The term "petroleum ether" refers to a fraction of b.p. 60–80°.

3-O-Acetyl-1,2-O-isopropylidene-α-D-glucofuranose. — To a solution of 3-*O*-acetyl-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose[18] (7.5 g) in methanol (30 ml) was added 0.8% aqueous sulfuric acid (30 ml). After 3.5 h at room temperature, the solution was saturated with sodium chloride, and extracted thoroughly with chloroform*. The dried (magnesium sulfate) extracts were concentrated to ~30 ml, and then heated to boiling. Petroleum ether was added to incipient turbidity, and the solution was allowed to cool. The title compound was obtained as fine needles, yield 5.8 g (89%), m.p. 125–126°; $[\alpha]_D$ −27.5° (*c* 1.8, ethanol); lit.[11] m.p. 122–123°, $[\alpha]_D^{20}$ −26.3° (ethanol).

3-O-Acetyl-1,2-O-isopropylidene-α-D-xylo-pentodialdo-1,4-furanose. — To a solution of 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose (2 g) in water (30 ml) was added sodium metaperiodate (18 g), and the solution was stirred for 15 min at room temperature. A further quantity of sodium metaperiodate (0.7 g) was added, and stirring was continued for 1 h. The solution was then extracted with chloroform (4 × 30 ml), and again (2 × 30 ml) after standing overnight. The dried (magnesium sulfate) extracts were combined and evaporated at 60°/<1 torr to a syrup (1.6 g) which was dissolved in ether, and the solution was dried over calcium sulfate. After 0.5 h, the i.r. spectrum of the solution showed strong absorption at 5.7 μm (C=O), but no absorption attributable to OH. The free aldehyde was obtained as a syrup; n.m.r. data: τ 0.38 (1-proton doublet, *J* 1 Hz, aldehyde H), τ 7.97 (3-proton singlet, OAc), τ 8.50, 8.68 (3-proton singlets, CMe_2).

Reaction of 3-O-acetyl-1,2-O-isopropylidene-α-D-xylo-pentodialdo-1,4-furanose with methylenetriphenylphosphorane. — Methyltriphenylphosphonium bromide (13 g) was added to a solution of sodium amide (1.4 g) in liquid ammonia (20 ml) under nitrogen. The ammonia was removed, and the residue was dissolved in dry ether (30 ml) and the solution heated for 30 min under reflux. To the bright-yellow solution of methylenetriphenylphosphorane was added dropwise, with stirring, a solution of 3-*O*-acetyl-1,2-*O*-isopropylidene-α-D-*xylo*-pentodialdo-1,4-furanose (4.5 g) in dry ether (30 ml). The reaction mixture became dark, and a brown, resinous precipitate was formed. When addition was complete, the mixture was filtered, and the filtrate was washed successively with dilute sulfuric acid and water, and evaporated to yield a syrup, which was chromatographed on silica gel, with 1:4 ethyl acetate–petroleum ether as eluant, to give two compounds. 3-*O*-Acetyl-5,6-dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**2**) was obtained as an oil, yield 0.31 g (7%), b.p. 154–157°/0.7 torr, $[\alpha]_D$ −13.1° (*c* 5, chloroform); R_F 0.7 (t.l.c., 1:4 ethyl acetate–petroleum ether); λ_{max}^{film} 5.65–5.80 μm (OAc and C=C), no absorption attributable to OH; n.m.r. data: τ 7.94 (3-proton singlet, OAc), τ 8.47, 8.67 (3-proton singlets, CMe_2).

Anal. Calc. for $C_{11}H_{16}O_5$: C, 57.9; H, 7.0. Found: C, 57.6; H, 7.1.

*In one experiment, potassium hydrogen carbonate was added until the pH of the solution was 8. This procedure caused acetyl migration and yielded 6-*O*-acetyl-1,2-*O*-isopropylidene-α-D-glucofuranose.

5,6-Dideoxy-1,2-*O*-isopropylidene-α-D-*xylo*-hex-5-enofuranose (**1**) was obtained as an oil, yield 0.20 g (5.5%), which crystallized from cold petroleum ether; m.p. 64–65°, $[\alpha]_D$ −64° (*c* 1.5, chloroform); lit.[3] m.p. 61–65°, $[\alpha]_D$ −51.5° (*c* 1.1, chloroform).

Methyl 4,6-O-benzylidene-2,3-dideoxy-3-C-methylene-α-D-threo-hexopyranoside (**3**). — To a solution of methylenetriphenylphosphorane [prepared, as described, from methyltriphenylphosphonium bromide (1.0 g) and sodium amide (0.11 g)] in ether was added a solution of methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*threo*-hexopyranosid-3-ulose[12,13] (0.5 g) in ether (40 ml), and the mixture was heated for 2 h under reflux. The product was isolated as already described, and recrystallized from petroleum ether; yield 0.3 g (60%), m.p. 107–107.5°, $[\alpha]_D$ +234° (*c* 0.8, chloroform); R_F 0.5 (t.l.c., 1:4 ethyl acetate–petroleum ether); λ_{max}^{KBr} 3.27 (vinylic C–H), 6.05 μm (C=C); n.m.r. data: τ 2.33–2.84 (5-protons, Ph), τ 4.42 (1-proton singlet, benzylidene-methine H), τ 6.65 (3-proton singlet, OMe).

Anal. Calc. for $C_{15}H_{18}O_4$: C, 68.7; H, 6.92. Found: C, 68.7; H, 6.94.

6,7,8-Trideoxy-1,2:3,4-di-O-isopropylidene-α-D-galacto-oct-6-enose (**4**). — To a solution (30 ml) of ethylidenetriphenylphosphorane [prepared from ethyltriphenylphosphonium bromide (3 g) and sodium amide (0.32 g)] in ether was added, at reflux temperature during 0.5 h, a solution of 1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-hexodialdo-1,5-pyranose[15,16] (2 g) in ether (30 ml). The product was isolated as described for compound **2**, to give a chromatographically (g.l.c. and t.l.c.) homogeneous oil, yield 1.5 g (72%). An analytically pure sample of compound **4** was obtained by distillation, b.p. ~95° (bath)/0.01 torr, $[\alpha]_D$ −117° (*c* 1.7, chloroform); R_F 0.8 (t.l.c., 2:3 ethyl acetate–petroleum ether); λ_{max}^{film} 6.04 μm (C=C); n.m.r. data: τ 4.0–4.7 (3-proton multiplet, H-1 and 2 vinyl protons), τ 8.31 (3-proton doublet, *J* 5 Hz, C-7 Me), τ 8.45, 8.57, 8.68 (3-, 3-, and 6-proton singlets, CMe_2).

Anal. Calc. for $C_{14}H_{22}O_5$: C, 62.2; H, 8.15. Found: C, 62.2; H, 8.40.

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Note

A convenient preparation of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside

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p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside is widely employed as a substrate for the assay of 2-acetamido-2-deoxy-β-D-glucosidase[1,2] (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase, EC 3.2.1.30). Various preparative routes have been described, but these suffer in general from low overall yields and are often time-consuming. The following method offers a rapid and more convenient alternative to that described by Leaback and Weissman[3].

EXPERIMENTAL

p-Nitrophenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucoside (**1**). — Sodium hydride (5.19 g of a 50% dispersion in oil, 108 mmoles) was washed by decantation with three portions (100 ml) of anhydrous light petroleum (b.p. 40–60°), and excess of solvent was removed under diminished pressure in a desiccator over anhydrous calcium chloride. Anhydrous methyl sulphoxide (30 ml) was added to the solid hydride, and the suspension was stirred magnetically under anhydrous conditions for 30 min before dropwise addition of a solution of *p*-nitrophenol (15.6 g, 112 mmoles) in anhydrous methyl sulphoxide (45 ml). After a further 30 min, a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[4,5] **1** (20.3 g, 55.6 mmoles) in anhydrous methyl sulphoxide (45 ml) was added slowly with continuous stirring. The reaction mixture was stirred for 20 h at room temperature, continuous stirring. The reaction mixture was stirred for 20 h at room temperature, and the product was precipitated by pouring on to a slurry of crushed ice and water (3 l) containing acetic acid (50 ml). The white, or buff, crystalline material was collected on sintered glass and washed with iced water until the washings were colourless. After being dried over calcium chloride *in vacuo*, the crude product (13.8 g) had m.p. 229–230°. Recrystallisation from methanol–dichloromethane (1:1) afforded white needles (12.2 g, 47%), m.p. 238°, $[\alpha]_D^{22}$ −48.8° (*c* 1.0, pyridine). [Purification of the acetate **1** is probably desirable, since traces of sulphoxide decomposition products are more readily removed at this stage than from the free glycoside.] Leaback and Weissman recorded a yield of 37–40% of acetate **1**, m.p. 236–239° (dec.), $[\alpha]_D^{20}$ −47° (*c* 0.5, pyridine).

p-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside. — The acetate **1** (12 g) was de-*O*-acetylated by the method of Westphal and Schmidt[6]. Thorough desiccation of the product *in vacuo* over phosphoric oxide for 4 days at room temperature afforded *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (8.1 g, 93%), m.p. 207–208°, $[\alpha]_D^{22}$ −14.7° (*c* 0.3, water); [lit., m.p. 210–212°, $[\alpha]_D^{20}$ −15° (*c* 0.5, water) (ref. 3); m.p. 214°, $[\alpha]_D^{16}$ −25.5° (pyridine) (ref. 7); m.p. 210°, $[\alpha]_D^{22}$ −18° (*c* 1.0, acetone–water, 1:1) (ref. 1)]. The overall yield from 2-amino-2-deoxy-D-glucose hydrochloride was 26%; *cf.*, 16–21% obtained by Leaback and Weissman[3].

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Note

Some considerations of the kinetics of acid hydrolysis of cellotriose

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The rate of the acid-catalyzed hydrolysis of cellotriose in concentrated sulfuric acid solutions was investigated by Freudenberg *et al.*[1,2] by following the change in the reducing power of the acid solutions with time. In interpreting the kinetics of the hydrolysis, they *assumed* that the specific rates of hydrolysis of the two D-glucosidic bonds are equal, but different from the rate for that in cellobiose. Their values for the rate constants have been checked, and certain shortcomings have been discussed[3].

More recently, Noto La Diega[4,5] made extensive measurements of the change in reducing power during the hydrolysis of cellotriose at various temperatures and concentrations of acid. Secondary reactions occurring in the acid solutions (decomposition of D-glucose, and its recondensation to di- and oligo-saccharides) required that he correct his experimental values. However, he used a rate equation based on the assumption that the rate of hydrolysis is the same for the two D-glucosidic bonds in cellotriose, but different from the rate for that in cellobiose. The calculated values for the degree of bond splitting agreed very closely with the corrected, experimental values.

The present paper discusses limitations in interpreting the kinetics of acid hydrolysis of a homotrisaccharide, namely, cellotriose, when only the change in reducing power is used as a measure of the hydrolysis. Also, the rate data, and the basic assumptions made by Noto La Diega in the interpretation of the kinetics of hydrolysis of cellotriose, are examined.

The kinetics of acid hydrolysis of cellotriose, based on the change in reducing power. — The reactions involved in the hydrolysis of cellotriose, and the corresponding kinetic reaction-scheme, are shown in Fig. 1, where [CT] = the concentration of cellotriose at any instant, [CB] and [CB^1] = the concentration of cellobiose at any instant, [G_1], [G_2], [G_3], and [G_4] = the concentration of D-glucose at any instant, k_2 is the known rate-constant for hydrolysis of cellobiose, and $k_3^{(1)}$ and $k_3^{(2)}$ are the hydrolysis rate-constants for the two D-glucosidic bonds in cellotriose.

The pertinent rate-equations (*cf.*, refs. 6 and 7) are as follows.

Case I

$$k_3^{(1)} \neq k_3^{(2)}$$

$$k_2 \neq k_3^{(1)}$$

$$k_2 \neq k_3^{(2)}$$

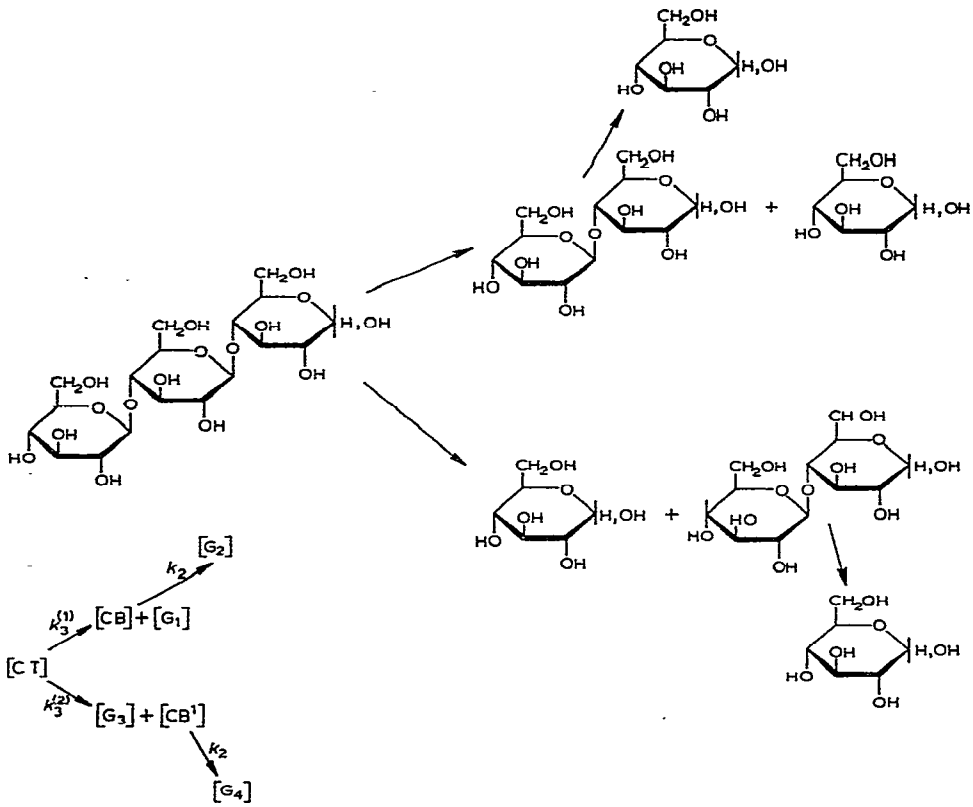


Fig. 1. Kinetic reaction-scheme for the hydrolysis of cellotriose.

Thus,

$$e^{k_2 t}(1-\alpha) = \frac{2k_2-(k_3^{(1)}+k_3^{(2)})}{2k_2-2(k_3^{(1)}+k_3^{(2)})} e^{-(k_3^{(1)}+k_3^{(2)}-k_2)t} - \frac{k_3^{(1)}+k_3^{(2)}}{2k_2-2(k^{(1)}-k_3^{(2)})}, \qquad (I)$$

where α is the ratio of the number of D-glucosidic bonds hydrolyzed to the number initially present, *i.e.* α expresses the degree of bond splitting. Its relation to the reducing power of the hydrolyzates is expressed by:

$$\alpha = \frac{R_t - R_0}{R_\infty - R_0},$$

where R_t is the value for the reducing power of the hydrolyzate, produced after a hydrolysis time of t, R_0 is that at $t = 0$, and R_∞ is that at infinite time.

Case II

$$k_3^{(1)} = k_3^{(2)} = k_3$$

$$k_2 \neq k_3$$

Thus,

$$e^{k_2 t}(1-\alpha) = \frac{k_2-k_3}{k_2-2k_3} e^{-(2k_3-k_2)t} - \frac{k_3}{k_2-2k_3}. \quad (2)$$

This is the rate equation used by Freudenberg *et al.* and by Noto La Diega, except that the latter author used corrected α values ($\alpha_{corr.}$) obtained from

$$\alpha_{corr.} = \frac{R_t - \Delta R - R_0}{R_\infty - R_0},$$

where ΔR is the correction used.

Cases III and IV

III	IV
$k_3^{(1)} = k_2$	$k_3^{(2)} = k_2$
$k_3^{(2)} \neq k_2$	$k_3^{(1)} \neq k_2$

Thus,

$$e^{k_2 t}(1-\alpha) = \frac{k_3^{(2)}-k_2}{2k_3^{(2)}} e^{-k_3^{(2)} t} + \frac{k_2+k_3^{(2)}}{2k_3^{(2)}} \quad (3)$$

and

$$e^{k_2 t}(1-\alpha) = \frac{k_3^{(1)}-k_2}{2k_3^{(1)}} e^{-k_3^{(1)} t} + \frac{k_2+k_3^{(1)}}{2k_3^{(1)}}. \quad (4)$$

Case V

$$k_3^{(1)} = k_3^{(2)} = k_2 = k$$

This represents the case when the kinetics of bond splitting in cellotriose would be simple, first-order, thus, $1-\alpha = e^{-kt}$, k being the first-order rate-constant for the hydrolysis of both cellotriose and cellobiose.

Equations *1*, *2*, *3*, and *4* can all be written in a general form, *viz.*,

$$e^{k_2 t}(1-\alpha) = Be^{Ct} + D, \quad (5)$$

in which the values for the constants B, C and D are not the same for Cases I, II, III, and IV.

As discussed in a previous paper[7], equation *5* can be brought into a form more suitable for computing the values of B, C, and D from experimental values of α when k_2 is known, namely,

$$\log_e \frac{\Delta[e^{k_2 t}(1-\alpha)]}{\Delta t} = -Ct + \text{constant}. \quad (6)$$

Thus, the fact that experimental values of α are found that satisfy equations *5* or *6* is not sufficient to distinguish between Cases I, II, III, or IV. This conclusion also

means that measurement of only the change in reducing power, with evaluation of the results by means of equation *5* or *6* does *not* allow computations of the specific rate of hydrolysis of either D-glucosidic bond in cellotriose unless it is assumed beforehand that the kinetics is according to Case I, II, III, or IV. Moreover, for Case I, even the sum $k_3^{(1)}+k_3^{(2)}$ can only be computed exactly if k_2 for cellobiose is known, and Cases III and IV are indistinguishable.

Examination of the data of Noto La Diega on the rate of hydrolysis of cellotriose. — Noto La Diega[4,5] used α values corrected for the effects of secondary reactions of D-glucose (degradation, and recondensation)[8]. In order to apply this correction to the rate data for the hydrolysis of cellotriose, the concentrations of the D-glucose and cellobiose formed on hydrolysis of cellotriose were needed. As these concentrations had not been measured, Noto La Diega calculated them, and these calculations, also, were based on the assumptions made for Case II.

The rate data of Noto La Diega have been tested by the use of equation *6*, on the basis of his k_2 and $\alpha_{corr.}$ values, and on the assumption that the kinetics of hydrolysis of cellotriose are according to Case II. Thus,

$$-\log_e \frac{\Delta[e^{k_2 t}(1-\alpha_{corr.})]}{\Delta t} = (2k_3-k_2)t + \text{constant.} \qquad (7)$$

Values for

$$-\log_e \frac{\Delta[e^{k_2 t}(1-\alpha_{corr.})]}{\Delta t}$$

have been plotted against t values, and some of these plots are shown in Fig. 2, from which it may be seen that the plots are not linear, as required by equations *6* or *7*: some of them show a marked curvature. At first glance, it would appear difficult to

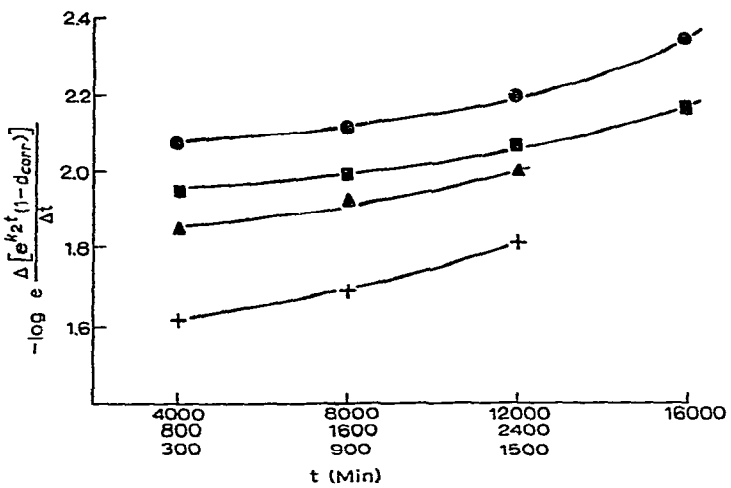


Fig. 2. Plots for rate of hydrolysis of cellotriose, according to *7*, where ● is 65% sulfuric acid at 6°; Δt = 4000 min; ■ 51% sulfuric acid at 18°; Δt = 4000 min; ▲ 65% sulfuric acid at 18°; Δt = 600 min; and + 51% sulfuric acid at 30°; Δt = 800 min.

reconcile this discrepancy with the apparently good agreement found by Noto La Diega between the calculated and the corrected-experimental α values. However, as the gradient of the plots, according to equation 7, is equal to $2k_3-k_2$, a large increase in the gradients results in only a small increase in the value of k_3. Thus, for the hydrolysis in 65% sulfuric acid solution at 6°, the gradient for the early period is 0.122×10^{-4}, and, for the later period, 0.381×10^{-4}, corresponding to k_3 values of 0.770×10^{-6} sec^{-1} and 0.995×10^{-6} sec^{-1}. In 51% sulfuric acid solution at 18°, the gradients are 0.85×10^{-4} (early period) and 2.62×10^{-4} (final period), corresponding to k_3 values of 5.41×10^{-6} sec^{-1} and 7.85×10^{-6} sec^{-1}. It should be noted that the k_3 values computed from the average gradients are close to those derived by Noto La Diega.

The considerable departure from linearity of some of the plots indicates that (*a*) the kinetics of hydrolysis of cellotriose does not conform to Cases I, II, III, or IV, and (*b*) the $\alpha_{corr.}$ values of Noto La Diega may not be entirely correct. However, even were these plots linear, the main issues [*i.e.*, whether (*a*) the two D-glucosidic bonds in cellotriose are hydrolyzed at the same rate, and (*b*) this rate differs from that of cellobiose] remain unsettled: they must be established by a method other than measurement of the change in reducing power. In recent work, Feather and Harris[9] used cellotriose-*1*-^{14}C, and followed the progress of hydrolysis by measuring its initial activity and that of the resulting cellobiose-*1*-^{14}C. They concluded that the D-glucosidic bond adjacent to the nonreducing end of the cellotriose molecule is hydrolyzed at about 1.5 times the rate for the bond adjacent to the reducing end, and at a rate nearer to that for cellobiose. Theoretical considerations[9,10] also lead to prediction of a faster hydrolysis of the D-glucosidic link at the nonreducing end of the cellotriose molecule.

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Note

Methylation of carbohydrates bearing base-labile substituents, with diazomethane–boron trifluoride etherate. III*. New syntheses of 4-*O*-methyl-D-galactose and 4,6-di-*O*-methyl-D-galactose

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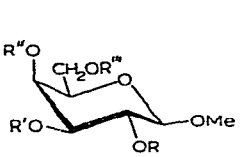
(Received October 29th, 1968)

It has previously been shown[1] that monosaccharides having base-labile substituents can readily be methylated by the use of diazomethane in the presence of a catalytic proportion of boron trifluoride etherate, without migration of the labile substituents. In our previous experiments, the stability of *O*-acetyl groups towards the action of the methylating reagents has been tested. We now report an extension of the method in which the reagents are employed with monosaccharides having *O*-benzoyl groups in the molecule.

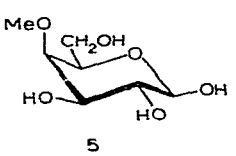
Bacon *et al.*[2] submitted methyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside (**2**) to the action of Purdie's reagents in an attempt to prepare the 4-*O*-methyl derivative, but methyl 2-*O*-methyl-3,4,6-tri-*O*-benzoyl-β-D-galactopyranoside was obtained instead; this result clearly indicated that an *O*-benzoyl group had migrated during the reaction, showing that *O*-benzoyl groups, as well as *O*-acetyl groups, are unstable in the alkaline medium of Purdie's reagents.

When our methylating reagent was applied to compound **2**, the expected methyl 2,3,6-tri-*O*-benzoyl-4-*O*-methyl-β-D-galactopyranoside (**3**) was readily formed. This product was debenzoylated with sodium methoxide in methanol to the hitherto-unknown methyl 4-*O*-methyl-β-D-galactopyranoside (**4**), which, upon acid hydrolysis, yielded 4-*O*-methyl-β-D-galactopyranose (**5**) whose physical properties were in agreement with values reported in the literature[3–5].

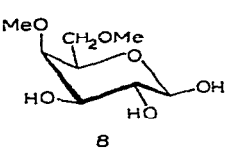
In order to obtain compound **2**, it was necessary to prepare methyl 2,3-di-*O*-benzoyl-β-D-galactopyranoside (**1**). This compound offered the opportunity to evaluate the methylating reagent with a partially benzoylated monosaccharide having



1 R=R'=Bz, R''=R'''=H
2 R=R'=R'''=Bz, R''=H
3 R=R'=R'''=Bz, R''=Me
4 R=R'=R'''=H, R''=Me
6 R=R'=Bz, R''=R'''=Me
7 R=R'=H, R''=R'''=Me



5



8

*For Part II, see ref. 1.

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two hydroxyl groups free. When compound **1** was treated with diazomethane–boron trifluoride etherate, methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-methyl-β-D-galactopyranoside (**6**) was obtained. This product failed to crystallize, but it was readily purified by high-vacuum distillation. Upon debenzoylation with sodium methoxide in methanol, compound **6** yielded methyl 4,6-di-*O*-methyl-β-D-galactopyranoside (**7**) having essentially the same physical properties as the product described in the literature[6]. Acid hydrolysis of **7** gave 4,6-di-*O*-methyl-D-galactose (**8**) having physical constants in accordance with reported values[6,7].

It has thus been proved that the new methylation method is effective with monosaccharides having *O*-benzoyl substituents, and does not cause migration of the *O*-acyl groups. Not only one but two hydroxyl groups can be methylated in one step.

EXPERIMENTAL

Melting points were determined with a Fisher–Johns block, and are uncorrected. I.r. spectra were recorded with a Perkin–Elmer "Infracord" spectrophotometer. N.m.r. spectra were recorded in deuterium oxide or chloroform-*d*, with a Varian A-60 n.m.r. spectrometer. Microanalyses were performed by A. Bernhardt (Mulheim-Ruhr, Germany). Solvents were removed under diminished pressure below 50°.

Methyl 2,3,6-tri-O-benzoyl-4-O-methyl-β-D-galactopyranoside (**3**). — Methyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside[2] (**2**) (2.13 g) was dissolved in dichloromethane (20 ml), and the solution was cooled to 0°; boron trifluoride etherate (0.08 ml) was added, and, while the temperature was kept at 0°, diazomethane in dichloromethane was added until a faint, yellow color persisted in the solution. After 30 min at 0°, a white solid (polymethylene) was filtered off, and the filtrate was evaporated. The crystalline residue (2.20 g) was recrystallized (twice) from ethanol, to give pure **3** (1.09 g), m.p. 135–136°, $[\alpha]_D^{20}$ +29.1° (*c* 1.9, chloroform). The i.r. spectrum showed no absorption in the hydroxyl region; n.m.r. data: τ 4.13 (1-proton quartet, $J_{1,2}$ 7 Hz, $J_{2,3}$ 10 Hz, H-2), τ 4.59 (1-proton quartet, $J_{3,4}$ 3 Hz, H-3), τ 5.33 (1-proton doublet, axial H-1 of β-D form); τ 5.35 (broad signal, H-6), τ 5.90 (unresolved signal, H-4 and H-5), τ 6.42 (OMe at C-1), and τ 6.45 (OMe at C-4).

Anal. Calc. for $C_{29}H_{28}O_9$: C, 66.91; H, 5.42. Found: C, 67.25; H, 5.50.

Methyl 4-O-methyl-β-D-galactopyranoside (**4**). — Compound **3** (915 mg) was dissolved in methanol (50 ml), and the solution was treated with M methanolic sodium methoxide (1 ml) and kept for 12 h at room temperature. The solution was neutralized with Dowex-50W ion-exchange resin, the solid was filtered off, and the filtrate was evaporated. The crystalline residue (490 mg) was recrystallized (twice) from absolute ethanol to give pure **4** (263 mg), m.p. 175–176°, $[\alpha]_D^{20}$ −39.9° (*c* 1.3, ethanol); n.m.r. data: τ 5.71 (1 proton doublet, $J_{1,2}$ 7 Hz, axial H-1 of β-D form), τ 6.45 (OMe at C-1), and τ 6.49 (OMe at C-4).

Anal. Calc. for $C_8H_{16}O_6$: C, 46.14; H, 7.74. Found: C, 46.31; H, 7.80.

4-O-Methyl-β-D-galactose (**5**). — Compound **4** (200 mg) was dissolved in M HCl, and the solution was kept for 2.5 h at 100°. When cool, it was neutralized with Dowex-1 resin, the solid was filtered off, and the filtrate was evaporated. The

crystalline residue (150 mg) was recrystallized from ethanol. Pure **5** (98 mg) had m.p. 215–217°, $[\alpha]_D^{20}$ +54.5 (5 min) → +82.3° (final, *c* 1.1, water); n.m.r. data: 5 min after dissolution, H-1 gave a doublet at τ 5.41 ($J_{1,2}$ 7.5 Hz) indicating the pure β-D anomer; 8 h later, the previous doublet had decreased and another doublet (H-1 of α-D form) at τ 4.71 ($J_{1,2}$ 3 Hz) had appeared. The OMe signal appeared at τ 6.46.

*Methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-methyl-β-*D*-galactopyranoside* (**6**). — Methyl 2,3-di-*O*-benzoyl-β-D-galactopyranoside[2] (**1**) (1.23 g) in dichloromethane (30 ml) was treated with boron trifluoride etherate (50 μl) and with diazomethane in dichloromethane (as described for the preparation of compound **3**). In this case, the residue (1.32 g) obtained after evaporation of the solvent failed to crystallize, and was purified by distillation (165–170°/10^{-3} torr). The hard, gummy product had $[\alpha]_D^{20}$ +63.8° (*c* 1.5, chloroform); the i.r. spectrum showed no hydroxyl absorption; n.m.r. data: τ 4.18 (1-proton quartet, $J_{1,2}$ 7 Hz, $J_{2,3}$ 10 Hz, H-2), τ 4.62 (1-proton quartet, $J_{3,4}$ 3 Hz, H-3), τ 5.37 (1-proton doublet, axial H-1 of β-D form), τ 6.05 (1-proton triplet, $J_{4,5}$ 3 Hz, H-4), τ 6.26 (unresolved signal, H-5 and H-6), τ 6.47 (OMe at C-1 and C-4), and τ 6.56 (OMe at C-6).

Anal. Calc. for $C_{23}H_{26}O_8$: C, 64.17; H, 6.08. Found: C, 64.32; H, 6.01.

*Methyl 4,6-di-*O*-methyl-β-*D*-galactopyranoside* (**7**). — Compound **6** (1.05 g) in methanol (50 ml) was treated with M methanolic sodium methoxide, and the solution was kept for 12 h at room temperature, and processed as described for compound **4**. The crystalline residue (520 mg) obtained by removal of the solvent was recrystallized from absolute ethanol; pure **7** had m.p. 139–140°, $[\alpha]_D^{20}$ −38.9° (*c* 1.3, water); n.m.r. data: τ 5.72 (1-proton doublet, $J_{1,2}$ 7 Hz, H-1 of β-D form), τ 6.45 (OMe at C-1), τ 6.49 (OMe at C-4), and τ 6.58 (OMe at C-6).

*4,6-Di-*O*-methyl-α-*D*-galactose* (**8**). — Compound **7** (100 mg) in M HCl (10 ml) was hydrolyzed as described for compound **5**. The crystalline residue (56 mg) obtained by removal of the solvent was recrystallized from methanol–ethyl acetate to yield pure **8**, m.p. 145–147°, $[\alpha]_D^{20}$ +128.8 (6 min) → +76.2° (final, *c* 1.3, water). When recrystallized from absolute ethanol, compound **8** showed m.p. 148–150°; the specific rotation was unchanged. N.m.r. data: 5 min after dissolution, τ 4.74 (1-proton doublet, $J_{1,2}$ 3 Hz, H-1 of α-D form), τ 6.48 (OMe at C-4), and τ 6.59 (OMe at C-6). After 8 h, the intensity of the H-1 signal had decreased, and another doublet, at τ 5.43 ($J_{1,2}$ 7.5 Hz, equatorial H-1 of the β-D form) had appeared.

ACKNOWLEDGMENTS

We thank Mr. J. J. Ferrer for the measurement of the i.r. and n.m.r. spectra, and the Consejo Nacional de Investigaciones Científicas y Técnicas (Buenos Aires) and FORGE (New York) for financial support.

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Note

Crystalline modifications of 1,6-anhydro-β-D-altropyranose, and two strongly levorotatory esters thereof

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1,6-Anhydro-β-D-altropyranose (**1**) can be crystallized either in the anhydrous or in the monohydrated form. Benzoylation of **1** has yielded a pure, syrupy tribenzoate (**2**) having $[\alpha]_D^{20}$ −309° (in chloroform) and a crystalline tris(*p*-nitrobenzoate) (**3**) having $[\alpha]_D^{20}$ −362° (in chloroform). Details of their preparation and properties are given.

EXPERIMENTAL

Some thirty years ago, it was reported[1] from this laboratory that the melting point of **1**, taken in a capillary tube, appeared to be about 135° (after considerable preliminary sintering). The compound, after being dried in a vacuum desiccator over calcium chloride and potassium hydroxide, was found to be anhydrous; it showed $[\alpha]_D^{20}$ −215° (*c* 1, water). Later[2], the value $[\alpha]_D^{20}$ −213.0 ±0.6° (*c* 2.5, water) was reported. Subsequently, Newth and Wiggins[3] recrystallised **1** from ethyl acetate, and obtained prisms having m.p. 58–59° and $[\alpha]_D^{19}$ −195° (*c* 1.2, water). Although those authors did not so state, we now know that their compound was a monohydrate; their rotation corresponds to −217° when calculated on an anhydrous basis. Pedersen[4] recrystallized **1** twice from ethyl alcohol, and reported m.p. 134–135° and $[\alpha]_D^{20}$ −211° (*c* 0.6, methanol). Jones and co-workers[5] have recently isolated **1** as the anhydrous modification, having m.p. 133° (slight preliminary sintering) and $[\alpha]_D$ −210° (*c* 1.0, water); analyses confirmed its composition.

1,6-Anhydro-β-D-altropyranose (**1**) *monohydrate.* — About fifteen years ago, one of us recrystallized 100 g of **1** from 300 ml of hot 95% ethyl alcohol and obtained 1,6-anhydro-β-D-altropyranose monohydrate as acicular prisms having m.p. 60–65° (when heated rapidly). The $[\alpha]_D^{20}$ was −193.4 +0.7° (*c* 2.5, water), which is equivalent to −215.0 ±0.8° for anhydrous **1**.

Anal. Calc. for $C_6H_{10}O_5 \cdot H_2O$: C, 40.00; H, 6.72; H_2O, 10.00. Found: C, 39.76; H, 6.91; H_2O (3.5 h at 57°, *in vacuo*), 10.36.

The crystals described immediately above remained constant in weight during

*Fellow in the Visiting Program of the National Institutes of Health, 1968–1969.

twenty-five days of comparatively humid weather in September. However, when the sample was crystallized from hot ethyl acetate (100 ml/g) in the following January (dry atmosphere), the anhydrous form of **1** was obtained as long, prismatic needles having m.p. 136–138° (*Anal.* Calc. for $C_6H_{10}O_5$: C, 44.44; H, 6.22. Found: C, 44.63; H, 6.26). These crystals remained constant in weight during three days under normal atmospheric conditions; but, when kept in a room where the relative humidity was 47%, they gained, in less than a month, 60.7% of the weight theoretically required for conversion into the monohydrate. When the crystals were then kept in a closed vessel containing a beaker of water, they deliquesced and gained almost 60% of their own weight in a week. When this material was re-exposed to the normal, low, ambient humidity of January, the syrupy mixture lost weight and became crystalline again.

Thus, depending upon the conditions, anhydride **1** crystallizes either as the anhydrous compound or as a monohydrate, and it can change from one form to the other quite readily.

1,6-Anhydro-β-D-altropyranose tribenzoate (**2**). — A solution of compound **1** (3 g, 19 mmoles) in anhydrous pyridine (60 ml) was cooled in ice and stirred, and to it was added, dropwise, 20 ml (173 mmoles) of benzoyl chloride. After 4 days at room temperature, the solution was found to contain only one carbohydrate derivative (t.l.c.; Silica Gel GF_{254}, E. Merck, Darmstadt, Germany; dichloromethane; visibilization by spraying with 10% sulfuric acid and then heating). The reaction mixture, processed by the procedure described by Fletcher[6], yielded about 25 g (theory, 8.8 g) of a syrup that contained the desired tribenzoate (**2**), together with a considerable proportion of benzoic anhydride and a small proportion of benzoic acid (t.l.c.; Silica Gel GF_{254}; 24:1 benzene–methanol). The syrup was freed of benzoic anhydride by column chromatography (silica gel, 70–325 mesh, E. Merck; dichloromethane), and of benzoic acid by shaking an ethereal solution of the syrup with aqueous sodium hydrogen carbonate. The yield of the final syrup was 6.6 g (75%). For analysis and determination of optical rotation, the 1,6-anhydro-β-D-altropyranose tribenzoate (**2**) was dried at 65° under high vacuum; $[\alpha]_D^{20}$ −309° (*c* 0.5, chloroform).

Anal. Calc. for $C_{27}H_{22}O_8$: C, 68.35; H, 4.67. Found: C, 68.43; H, 4.67.

1,6-Anhydro-β-D-altropyranose tris(p-*nitrobenzoate*) (**3**). — A solution of compound **1** (3 g, 19 mmoles) in 100 ml of anhydrous pyridine was cooled and stirred, and 20 g (106 mmoles) of *p*-nitrobenzoyl chloride was added in five portions during 1 h. The mixture was kept for 24 h at room temperature, and then processed as described[6] for **2**, to yield a syrup (12.9 g; theoretical, 11.3 g) that mainly contained a single compound. After standing for some days, the syrup crystallized spontaneously. The product (**3**) was recrystallized from acetone–ethyl ether, and then from ethyl acetate in the form of tiny, almost colorless needless: wt. 8.3 g (73%), m.p. 133–134°, $[\alpha]_D^{20}$ −362° (*c* 1.5, chloroform).

Anal. Calc. for $C_{27}H_{19}N_3O_{14}$: C, 53.21; H, 3.14; N, 6.90. Found: C, 53.13; H, 3.15; N, 7.05.

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Note

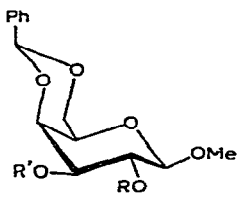
An improved procedure for the synthesis of 3-*O*-methyl-D-galactose

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In one of our research projects, there arose the need for 3-*O*-methyl-D-galactose (**5**). This compound has been obtained in several ways since the first synthesis by Reber and Reichstein[1]. It has been prepared by partial methylation of different substrates[2,3], by methylation of acetals of D-galactose[4], and from tetra-*O*-acetyl-D-galactoses of undetermined structure, by methylation followed by deacetylation[5]. The last four methods are very laborious, and involve chromatographic purification in several steps. Recently, **5** has been obtained by methylation of methyl and benzyl 2-*O*-benzyl-4,6-*O*-benzylidene-D-galactopyranosides followed by removal of the substituents, but syrupy material was obtained, and the yield was not disclosed[6]. Accordingly, we chose the procedure of Reber and Reichstein[1], who prepared **5** by methylation of methyl 4,6-*O*-benzylidene-2-*O*-*p*-tolylsulfonyl-β-D-galactopyranoside, followed by stepwise removal of the substituents.

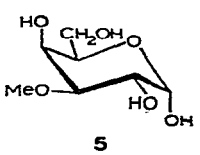


1 R = H ; R' = —COEt

2 R =  ; R' = —COEt

3 R =  ; R' = H

4 R =  ; R' = Me



5

We now report an improvement of this method by the use of dihydropyran as a protecting agent. Tetrahydropyran-2-yl ethers are stable to alkali, but are readily removed by acids, so that use of the 2-*O*-(tetrahydropyran-2-yl) group, instead of the 2-*O*-*p*-tolylsulfonyl group, simplifies removal of the substituents and leads, in one step, to the required ether **3**.

*Research member of the Consejo Nacional de Investigaciones Científicas y Técnicas.

Thus, methyl 4,6-*O*-benzylidene-3-*O*-(ethoxycarbonyl)-β-D-galactopyranoside[1] (**1**) was treated in *p*-dioxane with an excess of dihydropyran in the presence of a catalytic amount of *p*-toluenesulfonic acid, to yield methyl 4,6-*O*-benzylidene-3-*O*-(ethoxycarbonyl)-2-*O*-(tetrahydropyran-2-yl)-β-D-galactopyranoside (**2**) which, on treatment with potassium carbonate solution, produced methyl 4,6-*O*-benzylidene-2-*O*-(tetrahydropyran-2-yl)-β-D-galactopyranoside (**3**). Methylation of **3** with Purdie's reagents afforded methyl 4,6-*O*-benzylidene-3-*O*-methyl-2-*O*-(tetrahydropyran-2-yl)-β-D-galactopyranoside (**4**). Acid hydrolysis of **4** produced 3-*O*-methyl-α-D-galactose (**5**) directly, m.p. 143–145°, $[\alpha]_D^{19}$ +147.4→+107.0° (*c* 1.6, water), in accordance with reported values[1].

EXPERIMENTAL

General. — Melting points were determined with a Fisher–Johns hot-plate and are uncorrected. N.m.r. spectra were recorded with a Varian A-60 spectrometer in chloroform-*d* or deuterium oxide. Solvents were removed under diminished pressure below 50°.

Methyl 4,6-O-benzylidene-3-O-(ethoxycarbonyl)-2-O-(tetrahydropyran-2-yl)-β-D-galactopyranoside (**2**). — Methyl 4,6-*O*-benzylidene-3-*O*-(ethoxycarbonyl)-β-D-galactopyranoside[1] (**1**) (5.60 g) was suspended in a mixture of *p*-dioxane (12 ml) and dihydropyran (28 ml), and the suspension was cooled in an ice-bath. *p*-Toluenesulfonic acid (200 mg) was added, and the mixture was shaken until dissolution was complete while the temperature was kept below 15°. The solution was then kept for 48 h at 5°. Petroleum ether (b.p. 60–80°) was added, and the crystals that had formed were filtered off and washed with the same solvent. The crude product (4.60 g) showed traces of the starting compound (t.l.c.), and was purified by washing it with ether (2 × 15 ml) at room temperature and filtering off the solid after each washing. The product (4.10 g) showed no hydroxyl band in its i.r. spectrum; it was recrystallized (twice) from ethanol. Pure **2** (3.43 g) had m.p. 157–159°, $[\alpha]_D^{20}$ +138.4° (*c* 0.86, chloroform), and its n.m.r. spectrum was in accordance with the proposed structure.

Anal. Calc. for $C_{22}H_{30}O_9$: C, 60.26; H, 7.14. Found: C, 59.98; H, 7.12.

Methyl 4,6-O-benzylidene-2-O-(tetrahydropyran-2-yl)-β-D-galactopyranoside (**3**). — Compound **2** (3.40 g) was dissolved in warm methanol (130 ml), and the solution was cooled to room temperature. To this solution, 10% aqueous potassium carbonate (35 ml) was added, and the solid thus formed was redissolved by further addition of methanol (30 ml). The solution was then kept for 16 h at room temperature. The solvents were evaporated, and the crystalline residue was washed with cold water, the washings being discarded. The product (2.75 g) was recrystallized (twice) from ethanol; it had m.p. 164–165°, $[\alpha]_D^{20}$ +29.4° (*c* 0.85, chloroform). In the n.m.r. spectrum, the signals characteristic of the ethoxycarbonyl group [τ 8.70 (3-proton triplet, *J* 7 Hz, CH_3) and τ 5.17 (2-proton quartet, CH_2)] were absent.

Anal. Calc. for $C_{19}H_{26}O_7$: C, 62.28; H, 7.15. Found: C, 62.37; H, 7.19.

Methyl 4,6-O-benzylidene-3-O-methyl-2-O-(tetrahydropyran-2-yl)-β-D-galacto-

pyranoside (**4**). — Compound **3** (2.51 g) was mixed with methyl iodide (80 ml), and the mixture was heated for 24 h under reflux with the addition of silver oxide (12 g). The solid was filtered off, washed with acetone, and discarded. The filtrate was evaporated, and the crystalline residue was recrystallized (twice) from ethanol to yield pure **4** (2.26 g), m.p. 151–152°, $[\alpha]_D^{20}$ +78.2° (*c* 0.99, chloroform); the i.r. spectrum showed no hydroxyl band, and the n.m.r. spectrum was in agreement with the proposed structure.

Anal. Calc. for $C_{20}H_{28}O_7$: C, 63.14; H, 7.42. Found: C, 63.10; H, 7.52.

3-O-Methyl-α-D-galactose (**5**). — Compound **4** (2.20 g) was dissolved in acetone (62 ml), 2% sulfuric acid (23 ml) was added, and the mixture was refluxed for 2 h. The organic solvent was evaporated off, water (20 ml) was added, and the solution was re-evaporated; this procedure was repeated twice, to remove benzaldehyde completely from the solution. Water (20 ml) was added to the residue, and the solution was kept for 8 h at 100°. It was then neutralized with Dowex-3, and filtered, and the filtrate was evaporated. The residue, which was shown by paper chromatography to be identical with a sample of authentic material, crystallized from abs. ethanol; yield 870 mg, m.p. 127–133°. Recrystallization from the same solvent gave pure **5**, m.p. 143–145°, $[\alpha]_D^{19}$ +147.4 (10 min)→+107.0° (final, *c* 1.6, water). The n.m.r. spectrum showed that the compound was the α-D anomer, since, 10 min after dissolution, the H-1 signal appeared as a doublet at τ 4.71 ($J_{1,2}$ 3.5 Hz); after 7 h, the intensity of this signal had decreased, and another doublet (H-1 of the β-D anomer) at τ 5.38 ($J_{1,2}$ 7 Hz) had appeared. The *O*-methyl signal was at τ 6.53.

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Preliminary communication

A mild *a,e*-β-elimination reaction of some D-glucopyranosiduronate 4-sulfonates

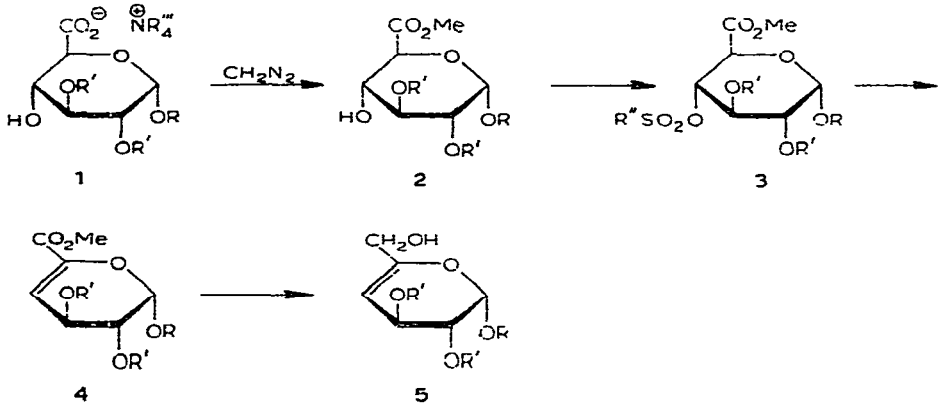
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The most characteristic structural feature of the oligosaccharides obtained by enzymic[1] or diazoalkane[2] degradation of mucopolysaccharides is the presence of 4,5-unsaturated 4-deoxyhexopyranosiduronate residues. In order to study the properties of this kind of structure, a simple synthesis from D-glucose has now been developed; it involves, as intermediates, D-glucopyranosiduronate 4-sulfonates protected by benzyl ether groups.

The methyl (or benzyl) 2,3-di-*O*-benzyl-α(or β)-D-glucopyranosiduronic acids were prepared[3] from D-glucose; their tetrabutylammonium salts (1) readily react with diazomethane (as do quaternary salts of the uronic groups of heparins[2]), resulting in the corresponding methyl ester (2).

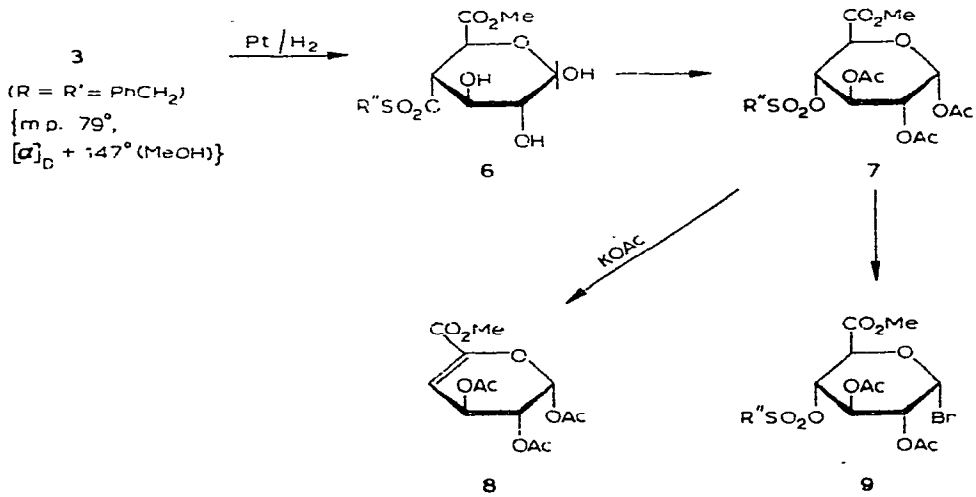


where R = Me, $PhCH_2$, or acyl; R' = $PhCH_2$ or acyl; R'' = Me or C_6H_4-Me-*p*; and R''' = alkyl.

On treatment with an alkali acetate in Ac_2O or Me_2SO, or with the theoretical amount of an alkali (*e.g.*, KOH or NaOMe) in methanol, the sulfonic ester 3 [*e.g.*, the methanesulfonate, R = R'' = Me, R' = $PhCH_2$: m.p. 63°, $[\alpha]_D^{25}$ +33° (*c* 0.9, chloroform)] gave, within a few minutes at 20°, an excellent yield of the corresponding 4,5-unsaturated 4-deoxypyranosiduronate 4, for R = Me, m.p. 68°, $[\alpha]_D^{25}$ +133° (*c* 0.9, $CHCl_3$), λ_{max}^{EtOH} 2.38 nm. N.m.r. data (60 MHz, in $CDCl_3$): low-field doublets at τ 3.95 (H-4), 5.18 (H-1), and 6.26 (H-2), a quartet at τ 5.73 (H-3), and singlets at τ 6.65 (OMe), 5.47, and 5.38 (2 benzyls);

$J_{1,2}$ 2.3 Hz, $J_{2,3}$ 7.0 Hz, and $J_{3,4}$ 2.6 Hz. O.r.d. data: (*p*-dioxane, 25°) $[\alpha]_{700}$ +123°, $[\alpha]_{500}$ +269°, $[\alpha]_{360}$ +623°, $[\alpha]_{300}$ + 1,353°, $[\alpha]_{257}$ +6,000° (max), $[\alpha]_{242}$ 0°, $[\alpha]_{240}$ −2,000°, and $[\alpha]_{220}$ −11,000°.

The olefin-forming reaction proved to be an axial–equatorial elimination[4], which can be carried out under very mild conditions[5]. Compound 6, having free hydroxyl groups on C-1, C-2, and C-3, can be obtained by catalytic hydrogenolysis of the benzyl protecting groups of compound 3; *e.g.*, compound 6, R″ = Me: m.p. 147°, $[\alpha]_D^{25}$ +72° (in MeOH, final value, 3 days). Acylation gave compound 7 (R″ = Me) m.p. 140°, $[\alpha]_D^{25}$ +58° (*c* 0.34, chloroform) ; by t.l.c. on Silica Gel with solvent A, namely, 3:7 (v/v) acetone–petroleum ether (b.p. 40–45°), R_F = 0.6. Further characterization was achieved by i.r. and n.m.r. spectroscopy.



Just as for the benzyl-protected derivatives, the 1,2,3-triacetate (7) of the 4-*O*-(methylsulfonyl)-D-glucopyranosiduronate gave, on treatment with anhydrous alkali acetate in Me_2SO or Ac_2O, the corresponding 1,2,3-triacetate (8) of the 4,5-unsaturated 4-deoxyhexopyranosiduronate in very good yield; *e.g.*, 8, R_F 0.45 (t.l.c. in solvent A), $[\alpha]_D^{25}$ +166° (*c* 0.23, chloroform), λ_{max}^{EtOH} 238 nm. Further characterization was achieved by i.r. and n.m.r. spectroscopy, and mass spectrometry.

By this method, the acylated, 4,5-unsaturated 4-deoxyhexopyranosiduronates such as 8 are readily prepared; they give, by saponification of the acyl protecting groups, the corresponding 3-deoxyhexulosonates. K. Meyer *et al.*[1] have described the formation of one of these compounds by cleavage of the unsaturated uronic acid–2-amino-2-deoxy-D-glucose disaccharide derivative obtained by bacterial (enzymic) degradation from hyaluronic acid and chondroitin sulfuric acids[6].

Furthermore, compound 7 proved to be a potential intermediate for preparing further unsaturated hexopyranosiduronate derivatives. For example, treatment with hydrogen bromide–acetic acid gave a very good yield of the bromide 9; it had m.p. 148°, $[\alpha]_D^{25}$ + 172° (*c* 0.49, chloroform), n.m.r.: $J_{1,2}$ 4.0 Hz.

This product (9) proved to be mainly the α-D anomer; it reacted readily (by the method of Koenigs and Knorr, or Zemplén *et al.*) with hydroxy compounds, to give the

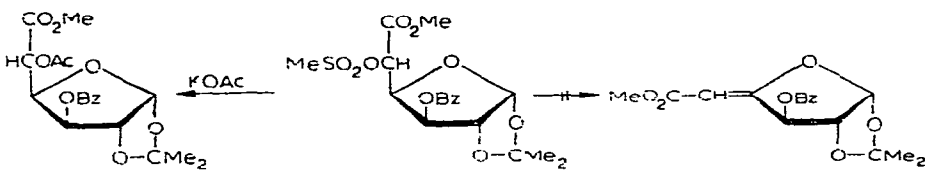
corresponding 4-methanesulfonate of the D-glucopyranosiduronate. Splitting of the methylsulfonyl group from the latter compounds led to the 4,5-unsaturated 4-deoxyhexopyranosiduronates (that is, di- or oligo-saccharides).

Saturation of the double bond[7] in the 4,5-unsaturated 4-deoxyhexopyranosiduronates by catalytic hydrogenation over Pd–C (as with the diazomethane degradation products of the heparins[2]) led mainly to the corresponding, substituted derivatives of 4-deoxy-D-glucopyranuronates[8].

Reduction of the carboxyl group in the unsaturated uronates (*e.g.*, **4**, R = Me, R′ = $PhCH_2$) with lithium aluminum hydride gave the 4,5-unsaturated 4-deoxy-α-D-glucopyranoside (**5**); R_F 0.4 (t.l.c. in solvent A), $[\alpha]_D^{25}$ +149° (*c* 0.88, chloroform), ν_{max}^{KBr} 3430 (OH) and 1685 (–C=C–) cm^{-1}; n.m.r. data (100 MHz, in $CDCl_3$); doublets at τ 5.03 (H-4) and 6.19 (H-1); quartets at τ 5.85 (H-3), τ 6.63 (H-2), and τ 6.04 (CH_2OH); and singlets at τ 5.47 and τ 5.38 (2 benzyls); $J_{1,2}$ 2.3 Hz, $J_{2,3}$ 6.3 Hz, and $J_{3,4}$ 3.0 Hz.

By similar β-elimination of the methanesulfonates, the corresponding 4,5-unsaturated 4-deoxy-β-D-glucopyranuronate was obtained. Compound **4**-β (R = Me, R′ = $PhCH_2$) had m.p. 111°; it was characterized by u.v., i.r., and n.m.r. spectroscopy, and by o.r.d. (max. $[\alpha]_{257-260}^{25}$ + 5,000° in *p*-dioxane). Moreover, this β-elimination is also appropriate for producing 4,5-unsaturated 2-(acylamido)-2,4-dideoxyhexopyranosiduronates

It is interesting that another type of *trans*-elimination of methanesulfonate in a rigid, substituted 1,2-*O*-isopropylidene-α-D-glucofuranuronate system, such as **10**, can *not* be carried out under similar conditions. Compound **10** had R_F 0.68 (t.l.c. in solvent A),



11 10

$[\alpha]_D^{25}$ –22.2° (*c* 0.81, chloroform). Further characterization was obtained by i.r. and n.m.r. spectroscopy, and microanalysis. Compound **10** proved to be stable in the presence of the theoretical amount of alkali (*e.g.*, KOH in methanol under reflux). The reaction with alkali acetates in Ac_2O led to the corresponding L-iduronate derivative (**11**), $[\alpha]_D^{25}$ +12.1° (in ethyl acetate). This is a useful way for producing 3-substituted L-iduronic acid derivatives.

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Preliminary communication

Carbohydrate fluorosulfates

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Study of the carbohydrate chlorosulfates has proven to be of value in synthesis, for example, of chlorodeoxy and of cyclic-sugar sulfate derivatives[1,2], and in elucidating conformational aspects of nucleophilic substitution at secondary carbon atoms of sugar derivatives[2,3]. These substitutions are characterized by an exceedingly high leaving-group ability of the chlorosulfate moiety, as shown by kinetic studies on a model series of alkyl chlorosulfates[4]. Fluoro substitution might further enhance this effect and study of carbohydrate fluorosulfates could be correspondingly useful. However, a previous attempt[5] to prepare this type of derivative directly by reaction between a sugar carbinol group and sulfuryl fluoride, analogous to the preparation of the chlorosulfates[1-3], was unsuccessful.

We now report the synthesis of some sugar fluorosulfates with several structural modifications: (*a*) OSO_2F on C-2 (hexopyranose); (*b*) OSO_2F on C-3 (hexofuranose); (*c*) OSO_2F on C-6 (hexopyranose); (*d*) a derivative having two fluorosulfate moieties, on C-2 and C-3 (hexopyranose). The method used involves the action of silver fluoride on known sugar chlorosulfates. The preparation of the last compound is described in detail.

Methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside 2,3-dichlorosulfate[2a] (2.36 g, 0.0054 mole) in methanol solution (100 ml) was treated with silver fluoride (5.4 g, 0.042 mole, Alfa Inorganics, Inc.) in water (20 ml) and the mixture was stirred at room temperature in the dark. Intermittent sampling and chromatographic (t.l.c.) testing for chlorosulfate by the butyl alcohol–aniline–pyridine spray reagent[6] showed that reaction was complete after 43 h. Filtration and concentration gave a syrup that was dissolved in chloroform, repeatedly washed with water, and then worked up in the usual manner, to yield methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside 2,3-difluorosulfate as a syrup, $[\alpha]_D$ +159.6° (*c* 1.7, chloroform). Anal.: Calc. for $C_7H_{10}O_8Cl_2F_2S_2$: C, 21.26; H, 2.55; Cl, 17.92; F, 9.64; S, 16.21. Found: C, 21.43; H, 2.73; Cl, 18.26; F, 9.81; S, 16.03. The n.m.r. spectrum of the product corresponded very closely to that of the reactant, indicating that no skeletal changes had taken place. The i.r. spectrum showed strong absorption at 1460 cm^{-1} and 1225 cm^{-1}, shifted from 1427 cm^{-1} and 1192 cm^{-1} compared with the reactant; the observed shifts in the i.r. frequencies correspond to those expected[7] for conversion of OSO_2Cl into OSO_2F.

By similar procedures the following fluorosulfates have been prepared from the corresponding chlorosulfates: methyl 3,4,6-trichloro-3,4,6-trideoxy-α-D-allopyranoside 2-fluorosulfate, 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-fluorosulfate, and 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose 6-fluorosulfate.

The primary fluorosulfate derivative decomposed on standing, precluding characterization by elemental analysis, but the former two fluorosulfates gave satisfactory analytical data. The relatively slow ion-exchange process at sulfur observed in these reactions is noteworthy

Further studies relating to the synthesis and reactions of the carbohydrate fluorosulfates are being undertaken, particularly as to their possible use as intermediates in the synthesis of the still difficultly accessible deoxyfluoro sugars.

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Announcement

A Euchem Conference on the Chemistry of Carbohydrates will be held at the University of Birmingham from 15–19 September 1969. Topics to be discussed include applications of physical methods, solvolytic and displacement reactions, and newer methods of synthesis. Further details from Dr. J.M. Webber, Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham 15.

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GAS–LIQUID CHROMATOGRAPHY OF HEXOPYRANOSIDES AND HEXOFURANOSIDES

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ABSTRACT

The behavior in g.l.c. of the acetates, trimethylsilyl ethers, and trifluoroacetates of 41 D-glucosides, D-galactosides, D-mannosides, and D-glucosiduronic acids in pyranose or furanose configuration was studied on several stationary phases in order to separate hexopyranoside and hexofuranoside derivatives. The relation between carbohydrate structure and retention time is discussed.

INTRODUCTION

Since McInnes *et al.*[1] reported the gas–liquid chromatography of methyl ether derivatives of carbohydrate in 1958, many investigations have been performed either on the acetates, trimethylsilyl ethers, or trifluoroacetates of carbohydrates[2–5].

In the study of the g.l.c. of methyl ethers of methyl D-xyloside isomers on Carbowax 6000 as stationary phase, Bishop and Cooper[6] showed that the β-pyranoside, α-pyranoside, β-furanoside, and α-furanoside were eluted in this order. The same authors also separated the isomers of methyl D-pentosides as their acetates or methyl ethers[2], and the isomers of methyl D-hexosides as their trimethylsilyl ethers[7]. The present investigation has been undertaken in order to separate hexopyranoside and hexofuranoside derivatives, and 41 alkyl and aryl D-glucosides, D-galactosides, D-mannosides, and D-glucosiduronic acids in pyranose or furanose form were chromatographed on six stationary phases as acetates, trimethylsilyl ethers, and trifluoroacetates.

RESULTS AND DISCUSSION

Per(trimethylsilyl) derivatives. — Table I shows the relative retention times of the per(trimethylsilyl) ethers of D-hexosides. Methyl α-D-mannopyranoside was the first one to be eluted of all the D-hexosides investigated with the six column packings. The α anomer of D-hexopyranosides was invariably eluted before the β anomer, whereas the β anomer of D-hexofuranosides was eluted before the α anomer. The order of the retention times of methyl D-glycosides was β-f < α-f < α-p < β-p for the methyl D-glucosides, and β-f < α-p < α-f < β-p for the methyl D-galactosides.

(Continued p. 340)

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TABLE I

RELATIVE RETENTION TIME IN THE GAS–LIQUID CHROMATOGRAPHY OF THE PER(TRIMETHYLSILYL) ETHERS OF HEXOSIDES[a]

Aglycon	*Ring*[b] *form*	*Anomer*	*Column material*						*Lit.*[c]
			1.5% *SE 30*	3% *SE 52*	2% *QF 1*	3% *NPG*	3% *BDS*	5% *UO*	
			Temperature (in degrees)[d]						
			170	200	160	180	160	200	
D-Glucoside									
Methyl	p	α	1.45	1.47	1.52	1.71	1.85	1.67	8
	p	β	1.56	1.54	1.65	1.81	2.02	1.85	8
	f	α	1.06	1.06	1.25	1.16	1.15	1.15	9
	f	β	1.04	1.02	1.23	1.14	1.01	1.11	9
Ethyl	p	β	1.95	1.70	1.74	2.20	2.13	1.88	10
	f	β	1.19	1.13	1.30	1.20	1.13	1.13	11
Phenyl	p	α	6.76	5.96	6.52	11.40	11.73	8.00	12
	p	β	8.57	7.00	8.60	16.80	19.47	10.38	12
	f	β	5.81	4.87	6.10	9.50	10.00	6.66	13
m-Tolyl	p	α	8.57	7.15	7.99	13.92	13.87	10.06	14
	p	β	10.86	8.52	10.30	20.20	23.60	12.75	14
	f	β	7.43	5.87	7.44	11.60	12.40	8.50	13
Guaiacyl	p	β	13.62	10.78	13.43	32.40	39.33	17.56	14
	f	β	10.86	8.09	10.32	20.80	22.67	12.63	13
(2-Naphthyl)	p	α	10.19 (200)	33.65	9.00 (190)	40.20 (200)	26.40 (190)	[e]	14
	p	β	11.81 (200)	38.96	10.90 (190)	54.40 (200)	38.93 (190)	[e]	14
	f	β	8.48 (200)	26.35	6.40 (190)	31.40 (200)	20.93 (190)	[e]	15

D-Galactoside									
Methyl	p	α	1.15	1.16	1.19	1.33	1.40	1.19	16
	p	β	1.32	1.30	1.57	1.61	1.90	1.50	17
	f	α	1.17	1.20	1.43	1.35	1.50	1.32	18
	f	β	1.03	1.00	1.13	1.08	1.23	1.09	18
Ethyl	p	α	1.19	1.17	1.14	1.20	1.13	1.09	16
	p	β	1.43	1.43	1.53	2.10	1.87	1.56	19
	f	β	1.10	1.09	1.11	1.10	1.07	1.06	20
Phenyl	p	α	5.24	4.74	5.55	9.50	9.67	6.28	21
	p	β	7.62	6.70	10.70	20.60	25.73	10.50	22
	f	β	5.14	4.61	6.65	9.30	10.69	6.50	23
m-Tolyl	p	α	6.90	5.96	6.95	11.50	12.56	7.88	24
	p	β	10.10	8.52	13.30	25.00	30.53	13.28	25
	f	β	6.76	5.91	7.89	11.50	13.47	8.28	26
Guaiacyl	p	α	9.14	7.91	12.27	20.40	22.93	11.63	24
	p	β	11.81	9.65	18.20	34.20	45.73	16.38	24
	f	β	8.86	7.65	14.20	20.20	25.87	11.94	26
D-Mannoside									
Methyl	p	α	1.00[f]	1.00[g]	1.00[h]	1.00[i]	1.00[j]	1.00[k]	27
	p	β	1.13	1.14	1.39	1.26	1.29	1.22	
	f	α	1.31	1.28	1.39	1.41	1.51	1.37	27
D-Glucosiduronic acid[l]									
Methyl	p	β	1.95	1.78	4.92	3.70	4.27	2.31	28
	f	β	1.33	1.30	2.44	2.20	2.67	1.44	29
(2-Naphthyl)	p	β	12.19 (200)	40.87	[m]	13.60 (200)	16.93 (190)	[e]	30
	f	β	9.43 (200)	25.91	[m]	13.40 (200)	17.07 (190)	[e]	15

[a]The values given are retention times relative to the trimethylsilyl ether of methyl α-D-mannopyranoside; the retention times of this compound were 1.05, 1.15, 0.90, 0.50, 0.75, and 1.60 min for *f*, *g*, *h*, *i*, *j*, and *k*, respectively. [b]p, Pyranose; f, furanose. [c]Reference of starting material. [d]Except as noted in parenthesis. [e]Was not eluted during 30 min at 200°. [l]As trimethylsilyl ester. [m]Was not eluted during 30 min at 190°.

TABLE II

RELATIVE RETENTION TIME IN THE GAS–LIQUID CHROMATOGRAPHY OF THE TRIFLUOROACETATES OF HEXOSIDES[a]

Aglycon	*Ring*[b] *form*	*Anomer*	*Column material*						*Lit.*[c]
			1.5% SE 30	*3% SE 52*	*2% QF 1*	*3% NPG*	*3% BDS*	*5% UO*	
			Temperature (in degrees)[d]						
			115	140	165	165	155	160	
D-Glucoside									
Methyl	p	α	1.00	1.05	1.09	1.11	1.10	1.03	8
	p	β	1.08	1.16	1.21	1.99	2.14	1.38	8
	f	α	0.86	0.93	0.93	1.06	1.10	0.98	9
	f	β	0.79	0.90	0.90	0.97	1.04	0.80	9
Ethyl	p	β	1.31	1.38	1.46	1.48	1.96	1.75	10
	f	β	0.95	0.92	1.04	0.87	0.85	0.90	11
Phenyl	p	α	8.55	7.34	4.64	2.26 (190)	2.74 (180)	*e*	12
	p	β	10.43	9.35	6.26	5.22 (190)	7.09 (180)	*e*	12
	f	β	7.19	5.70	4.12	2.09 (190)	2.65 (180)	*e*	13
m-Tolyl	p	α	14.00	10.33	5.39	2.61 (190)	3.04 (180)		14
	p	β	18.00	13.46	7.60	6.04 (190)	8.04 (180)		14
	f	β	10.48	7.57	4.76	2.35 (190)	2.77 (180)		13
Guaiacyl	p	β	30.95	21.21	10.84	8.70 (190)	12.35 (180)		14
	f	β	20.57	12.90	6.53	3.30 (190)	4.35 (180)		13
(2-Naphthyl)	p	α	9.62 (150)	12.34 (170)	7.64 (190)	*f*	*g*		14
	p	β	13.05 (150)	15.79 (170)	10.37 (190)	*f*	*g*		14
	f	β	7.29 (150)	9.25 (170)	6.83 (190)	*f*	*g*		15

Methyl	p	α	0.92	0.97	0.98	1.19	1.16	0.96	16
	p	β	1.03	1.27	1.30	2.50	2.78	1.70	17
	f	α	0.79	0.91	0.97	1.23	1.34	1.08	18
	f	β	0.78	0.80	0.89	1.05	1.13	0.80	18
Ethyl	p	α	1.00	1.03	1.13	1.04	1.00	1.08	16
	p	β	1.29	1.38	1.51	2.57	2.83	1.35	19
	f	β	0.90	0.84	0.92	0.96	1.00	0.95	20
Phenyl	p	α	8.60	6.76	4.39	2.83 (190)	3.20 (180)	[e]	21
	p	β	10.29	10.47	7.03	7.52 (190)	8.91 (180)	[e]	22
	f	β	7.33	5.98	4.64	2.70 (190)	3.03 (180)	[e]	23
m-Tolyl	p	α	11.24	9.30	5.12	3.13 (190)	3.30 (180)		24
	p	β	16.95	15.79	8.85	8.78 (190)	10.35 (180)		25
	f	β	10.86	8.60	5.67	3.13 (190)	3.87 (180)		26
Guaiacyl	p	α	20.57	16.17	7.65	4.52 (190)	5.04 (180)		24
	p	β	36.00	29.35	15.00	13.57 (190)	17.57 (180)		24
	f	β	16.86	12.62	7.26	4.13 (190)	5.04 (180)		26
D-Mannoside									
Methyl	p	α	1.00[h]	1.00[i]	1.00[j]	1.00[k]	1.00[l]	1.00[m]	27
	p	β	1.29	1.46	1.42	2.70	3.30	1.97	
	f	α	1.05	1.18	1.09	1.32	1.43	1.30	27
D-Glucosiduronic acid[f]									
Methyl	f	β	1.64	2.15	2.08	3.09 (190)	2.96		29
(2-Naphthyl)	f	β	4.52 (150)	6.92 (170)	2.87 (190)	[g]	[h]	[e]	15

[a]The values given are retention times relative to the trifluoroacetates of methyl α-D-mannopyranoside, the retention times of this compound were 1.05, 1.07, 0.82, 1.15, 1.15, and 1.00 min for *h*, *i*, *j*, *k*, *l*, and *m*, respectively. [b]p, Pyranose; f, furanose. [c]Reference of starting material. [d]Except as noted in parenthesis. [e]Was not eluted during 20 min at 200°. [f]As trimethylsilyl ester. [g]Was not eluted during 30 min at 250°. [h]Was not eluted during 30 min at 210°.

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TABLE III

RELATIVE RETENTION TIME IN THE GAS–LIQUID CHROMATOGRAPHY OF ACETATES OF HEXOSIDES[a]

Aglycon	*Ring*[b] *form*	*Anomer*	*Column material*						*Lit.*[c]
			1.5% SE 30	*3% SE 52*	*2% QF 1*	*3% NPG*	*3% BDS*	*5% UO*	
			Temperature (in degrees)						
			190	220	220	260	210	200	
D-Glucoside									
Methyl	p	α	1.12	1.08	1.10	1.12	1.17	1.08	8
	p	β	1.12	1.11	1.11	1.25	1.38	1.29	8
	f	α	1.02	1.05	0.84	0.98	0.98	1.07	9
	f	β	1.07	1.04	0.94	1.19	1.11	1.11	9
Ethyl	p	β	1.28	1.26	1.27	1.20	1.22	1.41	10
Phenyl	p	α	5.34	4.37	3.32	4.24	*d*	*e*	12
	p	β	6.22	4.84	4.07	5.65	*d*	*e*	12
	f	β	5.68	4.61	3.50	4.73	*d*	*e*	13
m-Tolyl	p	α	6.82	5.89	4.00	4.90			14
	p	β	8.15	6.63	5.00	6.50			14
	f	β	7.57	6.16	4.00	5.27			13
Guaiacyl	p	β	11.15	8.21	7.78	9.13			14
	f	β	10.27	8.16	5.79	7.50			13
2-Naphthyl	p	α	11.08	24.78	14.76	24.13			14
	p	β	12.23	27.16	18.65	33.15			14
	f	β	12.10	26.55	15.76	27.61			15

D-Galactoside									
Methyl	p	α	1.05	1.04	1.04	1.04	1.02	1.05	16
	p	β	1.05	1.06	1.07	1.23	1.35	1.08	17
	f	α	1.03	1.09	0.92	1.04	1.08	1.11	18
	f	β	0.99	1.02	0.92	0.99	1.05	1.05	18
Ethyl	p	α	1.22	1.21	1.15	0.95	0.88	1.08	16
	p	β	1.22	1.24	1.23	1.14	1.26	1.22	19
	f	β	1.15	1.16	0.96	0.96	0.95	1.15	20
Phenyl	p	α	5.13	4.74	4.35	4.35	[d]	[e]	21
	p	β	6.08	5.37	6.30	6.30	[d]	[e]	22
	f	β	5.41	4.79	4.62	4.62	[d]	[e]	23
m-Tolyl	p	α	7.30	6.00	5.00	5.00			24
	p	β	8.11	6.63	7.39	7.39			25
	f	β	7.36	6.26	5.33	5.33			26
Guaiacyl	p	α	9.39	7.90	7.28	7.28			24
	p	β	10.54	8.53	10.33	10.33			24
	f	β	9.93	8.00	7.61	7.61			26
D-Mannoside									
Methyl	p	α	1.00[f]	1.00[g]	1.00[h]	1.00[i]	1.00[j]	1.00[k]	27
	p	β	1.05	1.09	1.06	1.20	1.40	1.11	
	f	α	1.05	1.07	0.96	1.10	1.08	1.09	27
D-Glucosiduronic acid									
Methyl	p	β	0.95	0.89	0.91	0.87	1.11	1.02	29
Ethyl	p	β	1.01	1.00	0.97	0.90	1.06	1.12	

[a]The values given are retention times relative to the acetate of methyl α-D-mannopyranoside; the retention times of this compound were 0.74, 0.85, 0.82, 0.80, 7.20, and 8.80 min for *f*, *g*, *h*, *i*, *j*, and *k*, respectively. [b]p, Pyranose; f, furanose. [c]Reference of starting material. [d]Was not eluted during 30 min at 210°. [e]Was not eluted during 30 min at 200°.

These elution orders are in good agreement with those obtained by Smirnyagin *et al.*[7] on Carbowax 6000, polyphenoxy ether [*m*-bis(*m*-phenoxyphenoxy)benzene], and neopentylglycol sebacate.

The elution order of the aryl glycosides was β-f < α-p < β-p on all columns for the aryl D-glucosides and β-f < α-p < β-p on SE 30, SE 52, and NPG, and α-p < β-f < β-p on QF 1, BDS and UO for the aryl D-galactosides. The variations of retention time depend on the structure of the adsorbed material, as well as on the polarity of the liquid phase.

If the conformation of the methyl D-hexosides is assumed to be *C1*, some limited relationships may be established. The retention time was decreased by the presence of an axial methoxyl group at C-1 (*i.e.* the α anomer was eluted before the corresponding β anomer) or by that of a bulky axial substituent at C-2 or C-4. Thus, methyl α- and β-D-mannopyranosides were eluted before methyl α- and β-D-glucopyranosides, respectively, and methyl α- and β-D-galactopyranosides were eluted before methyl α- and β-D-glucopyranosides, respectively. Substitution of a bulky axial group at C-2 decreased the retention time more than was decreased by the same substitution at C-4, (*i.e.* methyl α- and β-D-mannopyranosides were eluted before methyl α- and β-D-galactopyranosides, respectively). These results are in agreement with the observation made by Ferrier[31] in the study of the trimethylsilyl ethers of pentopyranose on Apiezon L that axial substitution in the pyranose ring decreases the retention time.

Trifluoroacetyl derivatives. — Table II shows the relative retention times of the trifluoroacetates of D-hexosides. In contrast to the observation made in the study of the trimethylsilyl ether series, methyl α-D-mannopyranoside was not consistently eluted first. The α anomer of D-hexopyranosides was always eluted before the β anomer. The retention times of the D-hexofuranosides of the methyl and ethyl D-hexosides were usually smaller than those of the D-hexopyranosides, the exception being the methyl D-mannosides and methyl D-galactosides on the polar, liquid phases, NPG, BDS, and UO. The β-D-furanosides of the aryl D-glucosides and galactosides were eluted before the D-pyranosides, with some exceptions for the D-galactosides on QF 1 and BDS. The elution order of methyl D-glucosides was β-f < α-f < α-p < β-p on all phases. The elution order of methyl D-galactosides, was β-f < α-f < α-p < β-p on nonpolar, liquid phases (SE 30, SE 52, and QF 1), and β-f < α-p < α-f < β-p on polar, liquid phases (NPG, BDS, and UO). Introduction of an axial methoxyl group at C-1 into the methyl D-gluco-, galacto- and manno-pyranoside structures decreased the retention time, whereas introduction of a bulky axial substituent at C-2 or C-4 did not necessarily decrease the retention time. No relationship between conformation and retention time similar to that found for the trimethylsilyl ether series could be observed for the trifluoroacetates of methyl D-gluco-, D-galacto-, and D-manno-pyranosides.

Acetyl derivatives. — Table III shows the relative retention times of the acetates of D-hexosides. The α anomer of the D-hexopyranosides was generally eluted before the β anomer, but identical retention times were observed on SE 30 for both anomers

of methyl D-glucoside, and methyl and ethyl D-galactosides, respectively. No elution pattern was observed for the alkyl D-hexosides, but the aryl α-D-hexopyranosides were eluted before the β-D-furanosides. The α anomer of the D-glucofuranosides was eluted before the β anomer, but the reverse order was observed for the D-galactosides. The relationship between conformation and retention time was almost similar to that found for the trimethylsilyl ether series of the methyl D-hexopyranosides. The retention time was shorter when an axial methoxyl group was located at C-1, or a bulky axial group at C-2 or C-4, with the exception of methyl β-D-mannopyranoside being eluted from BDS after methyl β-D-glucopyranoside. The presence of a bulky axial group at C-2 decreased the retention time more than did the same substitution at C-4, although methyl β-D-mannopyranoside was not always eluted from SE 52, BDS, and UO before methyl β-D-galactopyranoside. These results differ from those observed by Ferrier[32] in the study of pentose tetraacetates on Apiezon L; namely that the presence of an axial substituent at any position other than C-1 increased the retention time, whereas the presence of an axial acetoxy group at C-1 decreased it.

EXPERIMENTAL

A Yanagimoto Gas Chromatograph GCG-550 FT equipped with hydrogen flame ionization detector was used. The carrier gas was nitrogen. The column was a stainless steel tube (1.5 m. × 3 mm. *i.d.*) packed with the following material: 1.5% silicon SE 30 on 60–80 mesh Chromosorb W (SE 30); 3% silicon SE 52 on 60–80 mesh Chromosorb W (SE 52); 2% silicon QF 1 on 60–80 mesh Chromosorb W (QF 1); 3% neopentylglycol succinate on 60–80 mesh Chromosorb G (NPG); 3% 1,4-butanediol succinate on 60–80 mesh Chromosorb G (BDS); and 5% Ucon oil 50 LB 550X on 60–80 mesh Chromosorb W (UO).

The D-hexosides were trimethylsilylated with hexamethyldisilazane and chlorotrimethylsilane in pyridine as described by Sweeley *et al.*[4], trifluoroacetylated with trifluoroacetic anhydride and sodium trifluoroacetate in acetonitrile, as described by Vilkas *et al.*[5], and acetylated with acetic anhydride and pyridine.

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REACTIVITY OF "V" AMYLOSE TOWARDS HYDROGEN CHLORIDE

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ABSTRACT

Gaseous hydrogen chloride catalyzes degradation both of "V" amylose hydrate and of a hydrated, amorphous amylose at 26° to give D-glucose and maltose. "V" Amylose dehydrate forms a molecular complex with gaseous hydrogen chloride at 48°/700 torr.

INTRODUCTION

Reaction between "V" amylose and hydrogen chloride, a volatile and acid molecule of small size, has been investigated as part of a continuing study of the chemical behavior of this form of amylose. Earlier papers have reported results of interactions of water and ammonia molecules towards the "V" amylose surface[1,2].

Dehydration of dried, granular starch by hydrogen chloride has been observed[3] at 70°. As amylopectin and other granular material are absent, amylose is considerably more homogeneous than native starch, as a substrate for chemical reaction. The X-ray diffraction pattern of "V" amylose indicates a helical structure[4,5]; interhelical and intrahelical channels are present within polymer crystals. Therefore, were selective reaction found between the carbohydrate polymer and hydrogen chloride, a correlation between chemical properties and structural details of the "V" amylose helix would become possible.

EXPERIMENTAL

Contact between hydrogen chloride and amylose was conducted, in the absence of air, in a vacuum system under controlled temperatures and pressures. The general features of the vacuum system have been described previously[2]. Hydrogen chloride was introduced into a reaction chamber containing samples of amylose; the apparatus was so designed that the reaction chamber could be disengaged from the manifold of the vacuum system.

Both "V" amylose dehydrate, having a helix packing-diameter of 13.0 Å, and "V" amylose hydrate, which has a packing diameter[1] of 13.7 Å, were investigated. These substances, together with amorphous amylose, were prepared according to

Nicolson *et al.*[1,2]. The "V" amylose dehydrate was obtained by drying the precipitate formed by reaction between a solution of amylose and butyl alcohol; "V" amylose hydrate was prepared by exposing the "V" amylose dehydrate to water vapor. The amorphous amylose was exposed to water vapor for the same period of time and at the temperature and pressure used to convert "V" amylose dehydrate into "V" amylose hydrate. Hydrogen chloride was contained in a cylinder (Matheson Co.), and was distilled through a cold trap prior to its contact with the amylose.

X-ray powder patterns were used to identify starting materials and reaction products. Samples were placed in thin-walled, glass capillaries; these were then sealed with Apiezon W wax. The camera radius was 57.3 mm; Cu radiation, which was used for diffraction, was filtered with a nickel foil. Intensity ratings listed in Table I for the "V" amylose–HCl complex were determined from a series of timed exposures.

The peracetates of D-glucose, maltose, and maltotriose are sharp-melting crystalline solids, and are easier to identify than the parent, unsubstituted compounds. Consequently, the products of reaction between amylose and HCl were acetylated, as the initial step in checking for degradation of amylose. Acetic anhydride (8 ml) and anhydrous pyridine (16 ml) were added to samples (2 g) of product, and mixture was stirred for 2 h at 90° and for 12 h at 25°. The mixture was poured into ice–water, to yield a water-insoluble syrup, which was dissolved in dichloromethane; the solution was successively washed with 3N hydrochloric acid, 10% aqueous sodium hydrogen carbonate, and distilled water, and evaporated *in vacuo* to a syrup. The dried syrup was examined for degradation compounds by t.l.c.; when such products were found, separation was attempted by column chromatography.

TABLE I

$\sin^2\theta$ VALUES (CuKα RADIATION) FOR THE "V" AMYLOSE DEHYDRATE–HCl COMPLEX PREPARED AT 48°/700 TORR

Hexagonal indices	*Intensity*	$Sin^2\theta$		*Hexagonal indices*	*Intensity*	$Sin^2\theta$	
		Obs.	*Calc.*			*Obs.*	*Calc.*
(100)	25	0.00463	0.00466	(301)	10	0.05113	0.05113
(110)	30	0.01403	0.01398	(311)	8	0.06971	0.06977
(200)	4	0.01864	0.01864	(400)	6	0.07423	0.07456
(111)	14	0.02303	0.02317	(410)	3	0.09796	0.09786
(201)	10	0.02784	0.02783	(411)	3	0.1071	0.1070
(210)	64	0.03258	0.03262	(330)	2	0.1260	0.1258
(300)	12	0.04199	0.04194	(510)	1	0.1467	0.1444
				(511)	1	0.1547	0.1536

Thin-layer chromatograms were obtained with Adsorbosil-3 (product of Applied Science Laboratories, Inc., State College, Pennsylvania), with 3:7 (v/v) ethyl acetate–benzene as eluant, and with Silica Gel G (distributed by Brinkman Instrument

Co., Great Neck, N. Y.), with methanol in benzene (2% by wt) as eluant. β-D-Glucose pentaacetate, maltose octaacetate, and a maltose heptaacetate were used as standards in spot identifications. Chromatographic columns were constructed with Biosil-A (chromatographic-grade silicic acid, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) and Magnesol–Celite; the identities of acetylated products were confirmed by mixed m.p. (with authentic samples) of the compounds recovered from the chromatographic columns. Identification of β-D-glucose pentaacetate, maltose heptaacetate, and maltose octaacetate indicated that D-glucose and maltose were present in the reaction products obtained from the hydrochlorination of amylose. A large proportion of material of high molecular weight remained unresolved by these separation techniques. The presence of a small proportion of maltotriose in certain samples was inferred, but positive identification was not made.

Certain of the products of the reaction of hydrogen chloride with "V" amylose dehydrate were analyzed for carbon, hydrogen, and chlorine; analyses were performed by Midwest Microlab, Inc., Indianapolis, Indiana.

RESULTS

Initial experiments indicated that, above room temperature, hydrogen chloride forms a complex with "V" amylose dehydrate. In order to determine the optimal conditions for preparation of the complex, reaction products were examined visually, by X-rays, and by chemical methods at 10-degree intervals between 28 and 68° and at pressures of 100 and 700 torr. Tan, brown, grey, violet, and black products were observed.

The results of the elemental analyses are listed in Table II, where the atomic proportions of the bulk products are expressed as formulas wherein carbon is fixed at 6 atoms, in order to compare findings relative to the hypothetical anhydro-D-glucose unit, $C_{6.00}H_{10.0}O_{5.00}$. Saturation of the "V" amylose dehydrate with hydrogen chloride occurred with increase in the temperature of reaction for a fixed length of time of reaction (48 h), as indicated by the chlorine content; the extent of incorporation of HCl was 0.29 molecule per 6 carbon atoms. The ratio of C to H in the reaction product decreased as the temperature of the reaction was increased. For the starting material, that is, the standard sample of "V" amylose dehydrate, the ratio was 6.00:11.5. An elemental analysis of "V" amylose dehydrate that had been subjected to additional drying (15 h) gave a ratio of C to H of 6.00:10.9. Chemical degradation to sugars of low molecular weight was not indicated; although thin-layer chromatograms were conducted for samples of "V" amylose dehydrate that had been exposed to hydrogen chloride (for 48 h at 700 torr, at 48 and 68°) and subsequently acetylated, no movement from the origin was shown on these chromatograms. Moisture was observed to collect in the neck of the reaction flask during one of the preparations (58°/700 torr).

An alteration of X-ray intensities occurred with the uptake of hydrogen chloride by "V" amylose dehydrate, indicating that a crystalline "V" amylose–HCl adduct

TABLE II

ELEMENTAL ANALYSES OF PRODUCTS OF REACTION BETWEEN "V" AMYLOSE DEHYDRATE AND HYDROGEN CHLORIDE DURING 48 H AT 700 TORR

Sample	*Temp. of reaction, degrees*	*% by weight*			*Atomic proportions in bulk product*
		C	*H*	*Cl*	
"V" Amylose dehydrate (standard)		44.35	7.12		$C_{6.00}H_{11.5}$
"V" Amylose dehydrate + HCl	38	42.60	7.15	3.84	$C_{6.00}H_{12.0}Cl_{0.18}$
	48	42.77	6.43	5.84	$C_{6.00}H_{10.7}Cl_{0.28}$
	58	45.15	5.95	6.44	$C_{6.00}H_{9.42}Cl_{0.29}$
	68	51.24	5.36	7.22	$C_{6.00}H_{7.48}Cl_{0.29}$

had been formed. The powder patterns of the reacted materials did not reveal changes in *d*-spacings between 28 and 68°. Therefore, a pseudohexagonal cell having a helix diameter of 13.0 Å was maintained by the crystalline amylose product. Some of the powder lines for the "V" amylose–HCl adduct appear beyond the largest $\sin^2\theta$ value observed for the starting material. The intensities of the reflections depended more on the temperature and pressure of the reaction than on the elapsed time of the reaction. At 28°, the diffraction diagram resembled that of the "V" amylose dehydrate. The best and sharpest detail was obtained from a sample prepared at 48°/700 torr for a reaction time of 48 h; the diffraction data ($\sin^2\theta$ values and intensities) are listed in Table I. A loss in crystallinity, as indicated by a fading of powder lines, occurred at temperatures above 48°. The data in Table I fit a hexagonal unit, and the best agreement between the calculated and the observed $\sin^2\theta$ values occurs for $a = 13.04$ Å and $c = 8.04$ Å. The limits of error estimated for both a and c are ± 0.07 Å.

In contrast to the experiments with "V" amylose dehydrate, chemical degradation to sugars of low molecular weight was indicated when hydrogen chloride was brought into contact with the "V" amylose hydrate at 26°. The reaction products were tan, gray, and violet in color. For a constant reaction-time, the X-ray diffraction patterns revealed that, by using the intensities of the powder lines as a guide, the crystallinity of the samples diminished as the pressure of hydrogen chloride was increased. A line-broadening effect also occurred, but the hexagonal lattice constant of 13.7 Å was, nevertheless, maintained. At pressure of 100 torr of hydrogen chloride, the powder pattern of the product resembled that of the starting material; at 700 torr, a pattern characteristic of amorphous material resulted. Caramelization occurred at 700 torr and an exposure time of 24 h. The results of chromatographic analysis of the acetylated reaction products are listed in Table III; products were formed under a pressure of hydrogen chloride of 525 torr, for a reaction time of 24 h, at 26°. Sample numbers refer to different samples treated under the same conditions. Hydrated, amorphous amylose was treated with hydrogen chloride under similar conditions of reaction, as a control; these results are also reported in Table III. Percentages are given on a weight basis. The percent recovery was determined from

an initial separation on a column of Magnesol–Celite; the percent of maltose consists of the sum of the recoveries of maltose heptaacetate and maltose octaacetate. No degradation products were observed when "V" amylose hydrate, not treated with hydrogen chloride, was acetylated.

TABLE III

CHROMATOGRAPHIC FRACTIONS FROM SYRUPS OF ACETYLATED REACTION PRODUCTS

Amylose sample	*No.*	*Recovery, %*	*Fraction, %*		
			D-Glucose	*Maltose*	*Higher molecular-weight oligomers*
"V", hydrate	1	95	13	7	70
	2	97	20	9	64
	3	97	10	5	84
	4	93	10	4	82
	5	87	12	4	71
Hydrated, amorphous	1	99	4	2	93
	2	99	22	21	55
	3	99	13	13	71
	4	96	26	21	50
	5	97	12	14	69

DISCUSSION

Both the exterior and the interior of the helix in "V" amylose are potentially open to attack by small molecules, as inter- and intra-helical channels are found in this structure. However, irregularities are present in crystals of "V" amylose, because of the polymeric nature of amylose. Irregularity or disorder occurs at crystal surfaces where chain folding exists, within the crystals at the ends of polymer molecules, and in strands of amylose that extend from, and occupy space between, polymer crystals[6]. If amorphous amylose is taken to represent the disordered regions of "V" amylose, the results of reaction with hydrogen chloride may be interpreted in terms of the ordered crystal-structure that is present.

Although, for the individual experiments listed in Table III, there is a considerable spread in the percentages of the D-glucose and maltose fractions recovered, a clear trend is found in the results; the ratio of the D-glucose to maltose fractions averages at 2:1 when "V" amylose hydrate is used as the substrate, but it is 1:1 for hydrated, amorphous amylose. If the disordered regions of the "V" amylose hydrate react in the ratio of 1:1, where the total "V" amylose hydrate ratio is 2:1, a very high D-glucose:maltose ratio results from chemical degradation occurring in the regular, or ordered, regions of the crystals of the "V" amylose hydrate. The spread in the percentages of the D-glucose and maltose fractions is greater for hydrated, amorphous amylose than for "V" amylose hydrate. Various degrees of short-range order are likely to be present in different samples of amorphous amylose.

Cyclohexaamylose serves as a model compound[7] for constructing a "V" amylose helix, if the helix pitch is altered from 0.0 to 1.33 Å. The complete structure of cyclohexaamylose has been determined by X-ray diffraction, and the glycosidic oxygen atom (O-4) is found on the inside of the ring of D-glucose residues[8]. Because a change from a ring to a turn of a helix requires only small adjustments between adjacent residues[7], the glycosidic oxygen atom has its unshared pairs of electrons exposed on the interior wall of a "V" amylose helix. There is sufficient room within the helix cavity to allow hydrogen chloride molecule to approach O-4. A proton from the acid could then be expected to associate with the electron pairs of the glycosidic oxygen atom. With cleavage of the O-4–C-1 bond taking place and a carbonium ion forming at C-1, water could hydrate the split fragments of the amylose chain. Protonated sugars would than be formed, which would subsequently release the proton. The process in the ordered regions in crystals of the "V" amylose hydrate is selective, in that, once initiated, successive D-glucose residues tend to split from the amylose chain; this accounts for the larger percentage of D-glucose found in the reaction product of the "V" amylose hydrate. Although the mechanism of bond-breaking is probably the same, the formation of a higher percentage of maltose is favored when hydrated, amorphous amylose is used as the starting material; with random geometry, cleavage is less likely to occur at successive linkage-points.

The role of water is critical in the cleavage of amylose to form sugars. Water aids ionization of molecular HCl after the latter has been adsorbed onto the carbohydrate substrate, and also effects hydration after carbonium-ion formation. Of interest is the low C:H ratio observed for the "V" amylose dehydrate that was used as the substrate for the reaction with HCl. It may be inferred that most of the hydrogen in excess of 10.0 in the 6.00:11.5 ratio of C to H is due to the presence of water in the "V" amylose dehydrate. Although a small proportion of residual butyl alcohol might also have been present in this sample, the effect of butyl alcohol on the C:H ratio would be small. The term "V" amylose dehydrate refers[1] only to water specifically involved in the change in helix packing-diameter from 13.7 to 13.0 Å. Molecules of water located in the disordered regions of the sample, or in the helix interior, cannot be detected by measurement of the lattice constant. Most important is the fact that the water present in the "V" amylose dehydrate is insufficient in proportion, or is not correctly located, to catalyze chemical breakdown to simple sugars.

Incorporation of hydrogen chloride by the "V" amylose dehydrate at 48° is similar to the formation of the "V" amylose–iodine complex. Changes in lattice constants do not occur when the adduct is formed[9,10], and corresponding colors are observed. Above 48°, an additional reaction occurs; this is apparent from the rise in the C:H ratio, as shown in Table II, and by the attendant loss in crystallinity of the reaction product. There are indications that water is formed by a dehydration process, and escapes from the sample. This high-temperature reaction was not investigated, except for testing for sugars. The latter were absent from the reaction product. It is noteworthy that a dehydration phenomenon has also been observed[3] between direct, granular starch and anhydrous hydrogen chloride at 70°.

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THE APPLICATION OF MASS SPECTROMETRY FOR IDENTIFICATION OF DERIVATIVES OF 2-SUBSTITUTED GLYCO-[1′,2′:4,5]-2-OXAZOLINES

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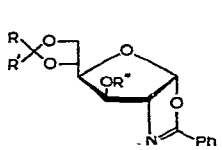
ABSTRACT

Based on the analysis of the mass spectra of a series of derivatives of 2-methyl- and 2-phenyl-glyco[1′,2′:4,5]-2-oxazolines, it has been shown that furanose and pyranose derivatives can be distinguished. The presence of an abundance of peaks due to the substituted oxazoline ring permits the ready identification of this class of monosaccharide derivative. Stereochemical differences in the compounds investigated have a small effect on the mass spectra.

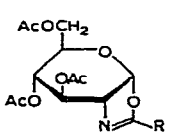
INTRODUCTION AND DISCUSSION

Derivatives of 2-substituted glyco[1′,2′:4,5]-2-oxazolines are convenient glycosylating reagents for synthesis of 2-amino-2-deoxyglycosides[1-3,8] having *trans* substituents at C-1 and C-2. Moreover, the oxazoline function can be used as a selective protecting group in synthetic chemistry of 2-acylamido-2-deoxy sugars[4,5]. For this reason, it is important to develop suitable and reliable methods for establishing the structure of this type of oxazoline derivative.

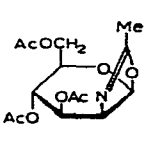
The mass spectra of 4,5-(2-deoxy-5,6-*O*-isopropylidene-α-D-glucofurano)-2-phenyl-2-oxazoline (**1**), 4,5-(3-*O*-acetyl-2-deoxy-5,6-*O*-isopropylidene-α-D-glucofurano)-2-phenyl-2-oxazoline (**2**), 4,5-(5,6-*O*-benzylidene-2-deoxy-α-D-glucofurano)-2-phenyl-2-oxazoline (**3**), 2-phenyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyrano)-2-oxazoline (**4**), 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyrano)-2-oxazoline (**5**), 2-methyl-4,5-(3,4,6-tri-*O*-acetyl-2-deoxy-β-D-mannopyrano)-2-oxazoline (**6**), 4,5-(4,6-*O*-benzylidene-2-deoxy-α-D-glucopyrano)-2-methyl-2-oxazoline (**7**), and 4,5-(4,6-*O*-benzylidene-2-deoxy-β-D-mannopyrano)-2-methyl-2-oxazoline (**8**) have been studied in the present work.



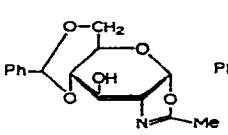
1 R = R′=Me, R″= H
2 R = R′=Me, R″= Ac
3 R = R″=H, R′=Ph



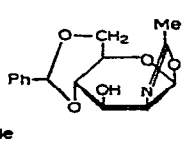
4 R = Ph
5 R = Me



6

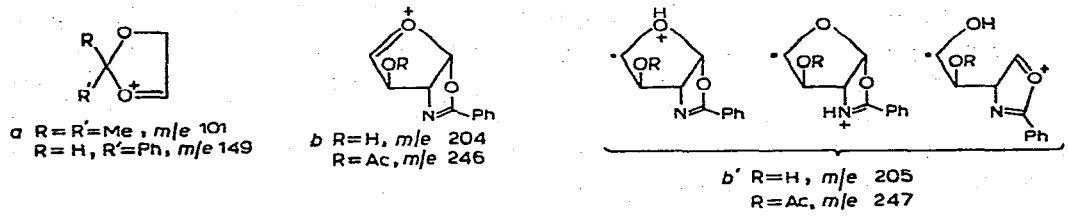


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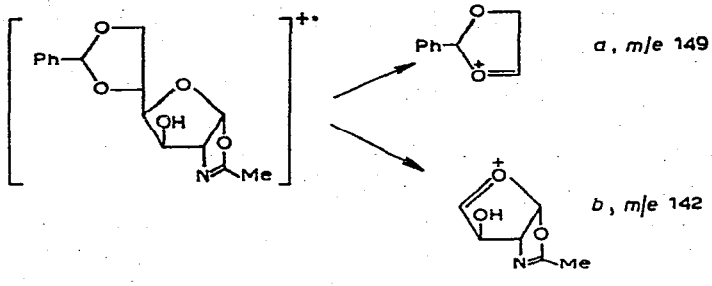
8

The spectra of all of the compounds investigated display a molecular peak of low or moderate abundance. The spectra of benzylidene derivatives **3**, **7**, and **8** contain an M−1 peak of higher intensity than the molecular peak (Figs. 1–3). The spectra **1–6** (Figs. 1 and 2) clearly show the cleavage of the oxide ring of the monosaccharide, with or without migration of a hydrogen atom to the charged fragment, and this is essential for determination of the ring size of the carbohydrate moiety. For example, the spectrum of **1** displays an abundant peak for ion *a* (*m/e* 101) as well as small peaks for ions *b* and *b'* (*m/e* 204 and 205, respectively) which confirm the furanose structure. A similar situation is observed in the mass spectrum of compound **2**, where the base peak corresponds to the ion *a* (*m/e* 101), and the peaks of the ions *b* and *b'* have *m/e* 246 and 247, respectively. In the spectrum of **3**, the peak of the ion *a* has *m/e* 149.

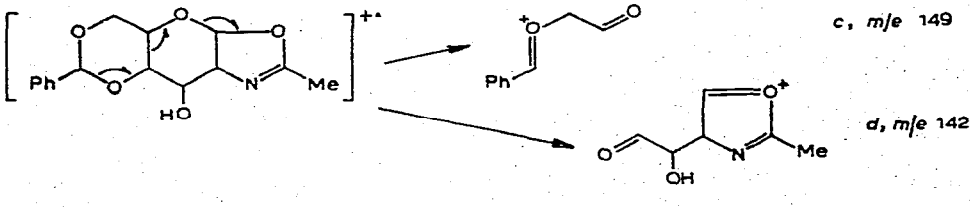


The spectra of compounds **4–6** display the peaks (M–73) of the ions arising by the expulsion of $\cdot CH_2OAc$, with *m/e* 256 for compounds **5** and **6** and 318 for compound **4**, which confirm the pyranose structure of these compounds.

In the case of the spectra of compounds **7** and **8**, the situation is much more complicated. The peaks with *m/e* 142 and 149 (Fig. 3) can be formed either by



cleavage of the C-4–C-5 bond in the compounds with the furanose structure (ions *a* and *b*) or by "H-cleavage"[6] of the pyranose derivatives to give ions *c* and *d*.



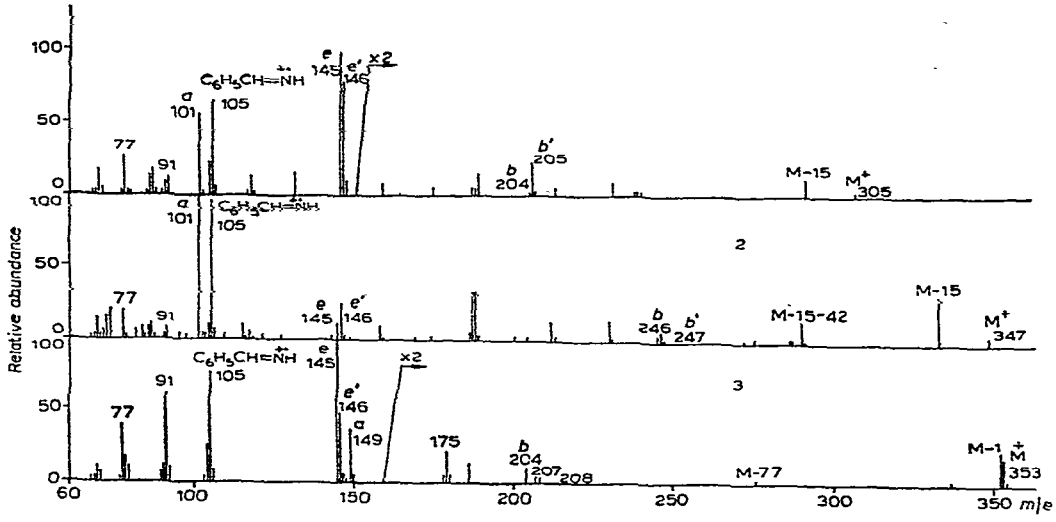


Fig. 1

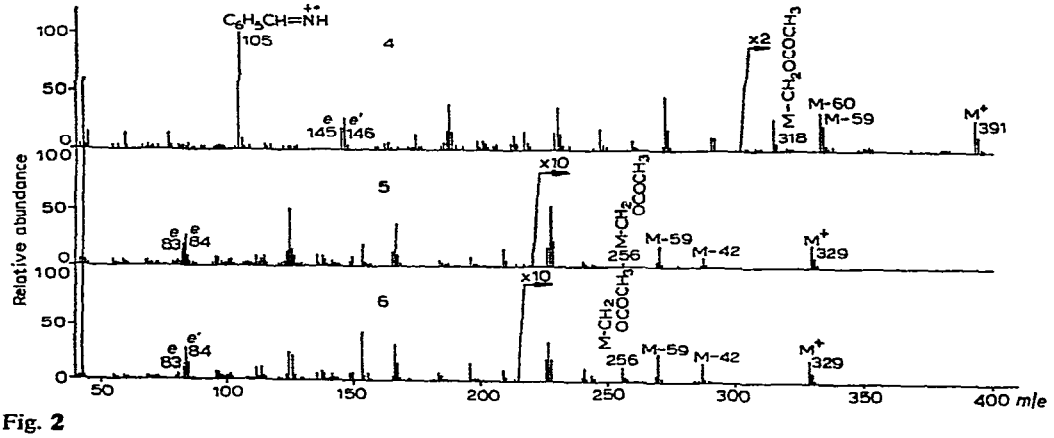


Fig. 2

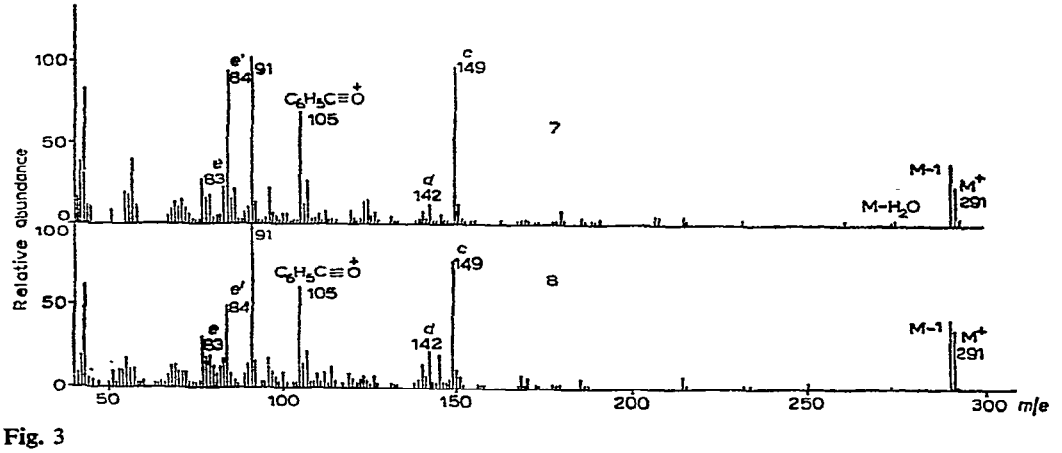
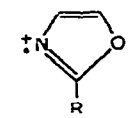


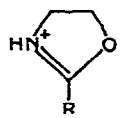
Fig. 3

However, the spectrum of the benzylidene derivative **3**, which contains a furanose ring, contains the peaks of the ions with *m/e* 179, 207, and 208, which retain the benzylidene group. These peaks are completely absent from the spectra of compounds **7** and **8**. This firmly establishes the pyranose structure of these compounds.

Another common fragmentation path for all of the compounds studied involves expulsion of the oxazoline ring in the form of ion radical *e* or ion *e'*, the peaks of which are usually of high abundance:



e, R = Ph, *m/e* 145
R = Me, *m/e* 83



e', R = Ph, *m/e* 146
R = Me, *m/e* 84

The structure of other fragments which contain a 2-oxazoline ring and which are due to the cleavage of different bonds of the sugar ring can be established by the study of deutero derivatives, and this will be reported elsewhere. The same is true for the fragments due to dehydration or elimination of acetyl groups.

The spectra of the pairs of stereoisomers **5** and **6** and **7** and **8** (Figs. 2*b*, *c* and 3*a*, *b*, respectively) are similar and differ only in the intensities of certain peaks; unequivocal conclusions on the effect of configuration on fragmentation under electron impact are not possible.

EXPERIMENTAL

The mass spectra were obtained with an MX-1303 spectrometer equipped with an all-glass system for direct introduction of the sample into the ion source[7]. The energy of the ionizing electrons was 30–40 *e*V, and the temperatures for the various compounds were **1**, 127°; **2**, 120°; **3**, 175°; **4**, 186°; **5**, 134°; **6**, 134°; **7**, 160°; and **8**, 170°.

Compounds **1–8** were synthesised by literature procedures[3,5].

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THERMODYNAMICS AND KINETICS OF D-GALACTOSE TAUTOMERISM DURING MUTAROTATION*

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(Received January 6th, 1969)

ABSTRACT

The tautomerism of D-galactose in water has been studied by using g.l.c. to separate per-*O*-trimethylsilyl derivatives of D-galactose tautomers formed during the mutarotation reaction. The initial, rapid stage of the biphasic mutarotation of α-D-galactopyranose is due to the formation of α-D-galactofuranose and β-D-galactofuranose. The slow phase is due to the formation of β-D-galactopyranose. The kinetic data of D-galactose tautomerism have been tabulated. The free-energy difference between pyranose tautomers (anomers) is primarily entropic. The free-energy difference between pyranose and furanose tautomers is distributed between relatively large and opposing enthalpic and entropic energies. It is concluded that the "transition state" for these tautomerization reactions is quite unlike the structure of any of the tautomers, and that, for the activation of a compound for tautomerization reactions, the same entropy and enthalpy exists for the formation both of furanoses and pyranoses.

INTRODUCTION

In the development of the chemistry of the carbohydrates, considerable emphasis has been directed toward the mutarotation reactions of the sugars[1–3]. As measured by optical rotatory techniques (o.r.), an acceptable explanation of the pseudo-first-order mutarotation exhibited by sugars having the *gluco*, *manno*, and *gulo* configuration is that the phenomenon is largely due to interconversion of the α- and β-D-pyranose tautomers. This explanation was further substantiated in a recent note[4] in which the formation of pyranoid tautomers of D-glucose and D-mannose was monitored by g.l.c. of their per(trimethylsilyl) ethers. These data were correlated with optical rotatory data, and agreement with both kinetic and thermodynamic data was found to be excellent. Therefore, it seemed that the g.l.c. procedure could be applied with confidence to the problem of the identity of tautomers formed during complex (multiphasic) mutarotation exhibited by sugars having the galactose, talose, and idose structures.

*Taken, in part, from a thesis, submitted by T. E. A. to Cornell University, in partial fulfilment of the requirements for the Ph. D. degree (1968).

During mutarotation of α-D-galactopyranose in pyridine, various tautomers which formed were isolated as their per(trimethylsilyl) ethers and subsequently characterized by chemical and instrumental techniques[5]. We now report direct evidence that the mutarotation of D-galactose *in water* is due entirely to the interconversion of pyranose and furanose structures of known[5] anomeric configuration.

RESULTS AND DISCUSSION

Fig. 1 shows a g.l.c. plot of per-*O*-(trimethylsilyl)ated tautomers of D-galactose at equilibrium in water at 25°. The peaks shown by these D-galactoside ethers are identical (superposable) with those previously isolated from a pyridine equilibrium

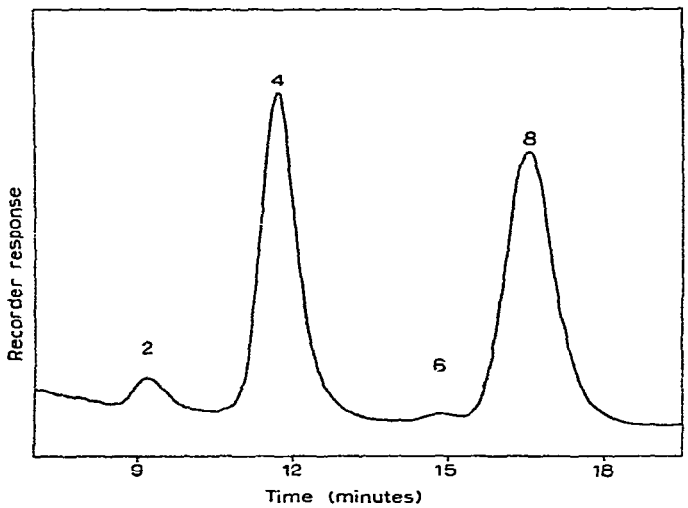
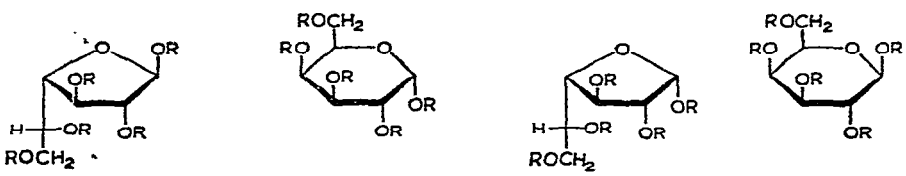


Fig. 1. Gas–liquid chromatogram of the per-*O*-(trimethylsilyl)ated derivatives of the tautomers of D-galactose formed at equilibrium optical rotation in water at 25°.

solution, and correspond, therefore, to the D-galactoses **1**, **3**, **5**, and **7**, in that order. The distribution of tautomers is **2**, 3.1; **4**, 32.0; **6**, 1.0; and **8**, 63.9%, a distribution different from that found at equilibrium in pyridine[5]. The total furanose content



(**1** + **5**) at equilibrium in water is 4.1%; this value is considerably less than the 12% furanose content estimated by Smith and Lowry[3] and the 9% furanose content

calculated by Sörensen[2]. Therefore, the relationship between the interconversion of these tautomers and the complex kinetics observed during the mutarotation of D-galactose needed to be established. This has now been accomplished by comparing the kinetics of mutarotation of α-D-galactopyranose in water, as monitored by o.r., with the kinetics of tautomerism as measured by g.l.c.

The optical rotation data for the mutarotation of α-D-galactopyranose in water have been described[3] by the equation:

$$\alpha^0 = Ae^{-m_1 t} + Be^{-m_2 t} + C$$

where m_1 is the "rapid phase" mutarotation coefficient and m_2 is the "slow phase" mutarotation coefficient. Table I compares the mutarotation coefficients obtained

TABLE I

THE MUTAROTATION COEFFICIENTS[a] AND THE PSEUDO-FIRST-ORDER RATE-CONSTANTS[a] FROM G.L.C., FOR α-D-GALACTOPYRANOSE IN WATER AT 15° AND 25°

	Slow phase		*Fast phase*	
Temperature, degrees	$M_1 \times 10^3$	k (*formation of β-pyranose*) $\times 10^3$	$M_2 \times 10^3$	k′ (*formation of β-furanose*) $\times 10^3$
15	5.18	4.89	34.5	35.0
25	13.1	13.0	53.3	56.5

[a]Calculated on the basis of log to base 10, and expressed in reciprocal minutes.

from the mutarotation of **3** in water at 298 and 288°K with the pseudo-first-order rate-constants obtained for the formation of the β-D-pyranose (**7**) and the β-D-furanose (**1**) from **3**, as measured by g.l.c. The rate constants from the g.l.c. kinetic data were calculated by using the equation

$$\log [X_t - X] = kt + \log [X_0 - X_\infty]$$

where X_t, X_0, and X_∞ are the relative, g.l.c. peak-areas of the tautomer X at time t, initially, and at equilibrium, respectively. Calculation of the rate constant for the formation of the β-D-furanose (**1**) from **3** was impossible to make directly, because of the extremely low concentration of **2** compared to that of **4**. Therefore, the samples were chromatographed at a lower attenuation, which moved the α-D-pyranoside peak well off the scale. The furanoside peak areas were then calculated relative to that of the β-D-pyranoside (**8**). The area of **8**, relative to the total, could be determined by using the rate constant for the formation of **7**; this was determined directly. Constants k and k^1 are the pseudo-first-order rate-constants for the formation of β-D-pyranose (**7**) and β-D-furanose (**1**), respectively.

The fact that the first-order mutarotation coefficient for the slow phase of mutarotation of α-D-galactopyranose is the same as the first-order rate-constant for the formation of β-D-galactopyranose as measured by g.l.c. supports the hypothesis[3]

that this part of the mutarotation is, indeed, due to an α-pyranose ⇋ β-pyranose tautomerism.

Within experimental error, the rapid phase can be completely explained on the basis of the formation of β-D-galactofuranose. Because the concentration of the α-D-furanose (**5**) is much smaller than that of **1** (**5** seldom exceeds 1% of the total content of tautomer), form **5** would not be expected to contribute much to the kinetics of mutarotation. However, the mutarotation of **3** in water containing 50 mole percent of pyridine yields a much higher concentration (16%) of furanoses. In this solution at 25°, the rate constants for the formation of **1** and **5** were almost identical (0.1637 and 0.1630 min^{-1}, respectively). If it is true that the rates of formation of **1** and **5** are the same, the properties of the mutarotation of **3** in water can be completely explained on the basis of the formation of **1**, **5**, and **7** at the expense of **3**. These experiments lead, for the first time, to an examination of the complex mutarotation of α-D-galactose by direct study of tautomers, of known structure, found.

The thermodynamics of equilibrium for the two reactions of D-galactose, namely, α-D-pyranose ⇋ β-D-pyranose, and α-D-pyranose ⇋ β-D-furanose, in water at 20° is shown in Table II. Just as for the results reported for D-glucose[4], the Gibbs

TABLE II

THE THERMODYNAMICS OF EQUILIBRIUM FOR THE TAUTOMERISM OF D-GALACTOSE AT 20°

	α-pyranose ⇋ β-pyranose	*α-pyranose ⇋ β-furanose*
ΔG	−410 cal. $mole^{-1}$	+ 1,590 cal. $mole^{-1}$
ΔH	− 25 cal. $mole^{-1}$	+ 7,520 cal. $mole^{-1}$
ΔS	+ 1.3 cal. $mole^{-1}deg.^{-1}$	+ 20 cal. $mole^{-1}deg.^{-1}$

free-energy difference between the α- and β-D-pyranose tautomers of D-galactose is primarily entropic in origin. However, the free-energy difference between the α-D-pyranose and β-D-furanose tautomers of D-galactose is a delicate balance between relatively large opposing enthalpic and entropic energies. Also, the α ⇋ β-pyranose tautomerism of D-mannose is, primarily, enthalpically controlled. Therefore, the tautomerism of sugars involves a wide range of enthalpic and entropic interactions. Because of the similarity to structural tautomerism, it would be expected that a wide range of enthalpic and entropic interactions would govern equilibria between sugar conformers; this would make difficult an extrapolation of the thermodynamic properties of one conformational sugar reaction to that of another.

The thermodynamics of activation for the reactions of α-pyranose ⇋ β-pyranose and α-pyranose ⇋ β-furanose are shown in Table III. For simplicity, the reactions are assumed to be monomolecular, first-order, reversible reactions. With respect to the mechanism of the tautomerism reactions of D-galactose, the data in Tables II and III contain two important pieces of information: (*1*) the fact that the transition state is significantly lower in entropy than either the pyranose or furanose tautomers

indicates that the transition state is quite different from either of the tautomers, and (*2*) the transition state for the activation of α-pyranose has, within experimental error, the same entropy and enthalpy for the formation of both the β-furanose and the β-pyranose form.

TABLE III

THE THERMODYNAMICS OF ACTIVATION[a] FOR THE TAUTOMERISM OF D-GALACTOSE AT 20°

	α-pyranose $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ *β-pyranose*		*α-pyranose* $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ *β-furanose*	
	k_1	k_2	k_1	k_2
k at 15°	0.00326	0.00163	0.0022	0.0328
k at 25°	0.00866	0.00434	0.0050	0.0515
ΔG	+22,115	+22,517	+22,412	+20,925
E_a	+16,679	+16,722	+15,637	+ 7,714
ΔS	−18.6	−19.8	−23	−45

[a]Rate constants are log to base e, ΔG and ΔH are in cal. mole^{-1}, and ΔS is in cal. mole^{-1}deg.$^{-1}$.

Thus, it appears that the four-component tautomerism of D-galactose may involve a common transition state or intermediate. The acid–base[6,7] catalysis, the complex solvent-effects[8–10], the low entropy of the transition state, and the common intermediate observed for the mutarotation of sugars indicate that the transition state is, most probably, a sugar–solvent or sugar–catalyst complex. In the case of D-galactose tautomerism, this complex does not appear to depend upon the equilibrium thermodynamics of tautomerism; more probably, it depends upon an interaction with the solvent. Hence, we consider that the nature of the medium (solvent and catalysts) is the most important parameter governing the tautomerism of the sugars in solution.

EXPERIMENTAL

The trimethylsilylation and g.l.c. procedures and the mathematical analysis have been described previously[4,5,12].

The rapid-phase mutarotation coefficient was calculated according to the procedure described by Isbell and Pigman[11].

ACKNOWLEDGMENTS

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4-DEOXY URONIC ACIDS

PART II*. THE ANOMERS OF METHYL (METHYL-4-DEOXY-D-*xylo*-HEXOPYRANOSID)-URONATE

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ABSTRACT

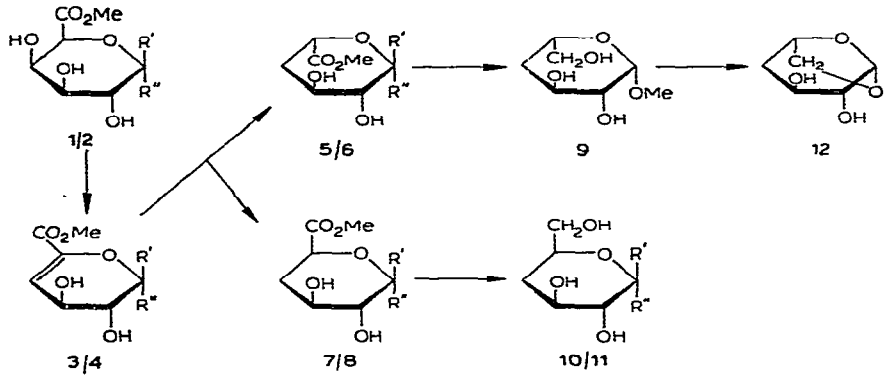
Starting from the anomers of methyl (methyl D-galactopyranosid)uronate the corresponding $\Delta_{4,5}$ unsaturated uronic acid derivatives, the anomeric methyl (methyl 4-deoxy-L-*threo*-hex-4-enopyranosid)uronates, have been prepared by a base-catalyzed elimination reaction. Factors influencing the yield in this reaction have been studied with the α anomer. Catalytic hydrogenation of methyl (methyl 4-deoxy-β-L-*threo*-hex-4-enopyranosid)uronate in dry methanol gave methyl (methyl 4-deoxy-β-L-*arabino*-hexopyranosid)uronate and methyl (methyl 4-deoxy-α-D-*xylo*-hexopyranosid)uronate in a ratio of 86 to 14. Under similar conditions, the unsaturated α anomer gave the corresponding 4-deoxy-α-L-*arabino* and 4-deoxy-β-D-*xylo* derivatives in a ratio of 5 to 95. These ratios are discussed considering stereochemical factors. The methyl 4-deoxyuronates were characterized by their crystalline amides, and their structures were confirmed by examination of the 100-MHz n.m.r. spectra and by reduction to the corresponding methyl 4-deoxy hexosides.

INTRODUCTION

The hydrogenation of methyl (methyl 4-deoxy-β-L-*threo*-hex-4-enopyranosid)-uronate (**3**) in dry methanol gives two crystalline products. By examination of the n.m.r. spectrum and by conversion to 1,6-anhydro-4-deoxy-L-*arabino*-hexopyranoside (**12**) the main product was shown to be methyl (methyl 4-deoxy-β-L-*arabino*-hexopyranosid)uronate (**5**). The minor product was assumed to be the C-5 epimer of **5**, methyl (methyl 4-deoxy-α-D-*xylo*-hexopyranosid)uronate (**7**), since the hydrogenation of **3** creates a new asymmetric center at C-5[1]. Confirmation of this proposed structure was recently provided by Cook and Overend[2] who described the synthesis of 4-deoxy-L-*arabino*-hexose and 4-deoxy-D-*xylo*-hexose using the same sequence of reactions. They did not, however, isolate the 4-deoxyuronic acid derivatives, but separated the epimeric methyl 4-deoxyhexopyranosides **9** and **10** after reduction of the mixture of the deoxyuronic acid esters with lithium aluminum hydride.

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This paper presents the synthesis of the 4-deoxy-L-iduronic acid derivative **5** and of the hitherto unknown 4-deoxy-D-glucuronic acid derivatives **7** and **8** and discusses their characterization.



1,3,5,7,10 R' = H ; R" = OMe

2,4,6,8,11 R' = OMe ; R" = H

DISCUSSION

Since consistent yields of the unsaturated uronic acid derivative **3** from methyl (methyl α-D-galactopyranosid)uronate[3,4] (**1**) could not be obtained, the reaction was further studied. The optimal reaction time for the formation of **3** was determined by u.v. measurements at 235 nm[3,4]. The results show that the maximum yield is obtained after about 40 min, in agreement with the time previously reported[3,4]. Activation by the ester group is necessary for the elimination to take place[3,4], since methyl α-D-galactopyranosiduronic acid was recovered quantitatively after being subjected to the same conditions. Qualitative thin-layer chromatography showed that compound **1** was saponified at a rate much faster than that of **3**, the ester group of the latter compound being stabilized by conjugation with the α,β double bond. Moisture in the solvent or in the starting material therefore reduces the yield.

Since the elimination reaction gives 1 mole of water per mole of substrate, the maximum yield of **3** cannot be raised above 50%, even under optimal conditions; and the highest yield obtained was 49.4%. In an attempt to bind the water formed during the reaction, magnesium methoxide was used instead of sodium methoxide, since the magnesium hydroxide formed is insoluble in methanol. The reaction mixture became, however, a gelatinous mass which could not be redissolved by addition of more methanol, even at a concentration of magnesium methylate of 0.25M, and the yield obtained was very poor. However, addition of a small quantity of 0.5M magnesium methoxide to the sodium methoxide solution prior to reaction gave usually yields of about 40%. Addition of methyl acetate in a 15-fold molar excess, or of dimethyl oxalate in a 2-fold molar excess, increased the yields to 48–49%, thus indicating that the esters effectively remove the hydroxyl ions which are already present as impurities, but fail to bind the water formed by the reaction.

Substitution at C-4 would facilitate the elimination reaction, because oxy functions are better leaving groups than the hydroxyl group itself[5], and because the by-product of the elimination is not water. The fully methylated derivative of **1** underwent almost quantitative elimination[4], and the unsaturated derivative **3** was obtained in a yield of 62% from the 2,3,4-tri-*O*-acetyl derivative of **1**. In this case, the elimination of acetic acid competes with the deacetylation at C-4. Under the conditions used, about 24% of the acetate underwent elimination prior to deacetylation at C-4, and the relative rate of the two competing reactions was about 1 to 3.

Methyl (methyl 4-deoxy-α-L-*threo*-hex-4-enopyranosid)uronate (**4**) was prepared by deacetylation[6] of its 2,3-*O*-diacetyl derivative, obtained from methyl (methyl β-D-galactopyranosid)uronate (**2**) by a method which will be described in a subsequent paper. The unsaturated uronic acid derivatives **3** and **4** were dissolved in dry methanol and hydrogenated at room temperature in presence of 10% palladium-on-charcoal. The hydrogen uptake ceased after 30 min, one mole of hydrogen being absorbed in both cases. Samples of the syrupy products were analyzed by g.l.c. as the per(trimethylsilyl) derivatives[7]. Two peaks were observed for each compound. The ratio of the products **5** and **7** obtained from **3** was 86 to 14, and that of the products **6** and **8** obtained from **4** was 5 to 95. The main derivative obtained from **3** was identified as methyl (methyl 4-deoxy-β-L-*arabino*-hexopyranosid)uronate (**5**)[1] and characterized as the crystalline amide **13**. Reduction of **5** with lithium aluminum hydride in dry tetrahydrofuran gave syrupy methyl 4-deoxy-β-L-*arabino*-hexopyranoside (**9**)[2]. The main crystalline product of the acid hydrolysis of **9** was shown to be 1,6-anhydro-4-deoxy-L-*arabino*-hexopyranose (**12**)[1,2]. The minor product obtained by hydrogenation of **3** was characterized by a crystalline amide **14**. The expected structure, methyl (methyl 4-deoxy-α-D-*xylo*-hexopyranosid)uronate (**7**), was confirmed by examination of the 100-MHz n.m.r. spectrum (Table I), as well as by

TABLE I

N.M.R. SPECTRAL DATA (100 MHz) OF METHYL (METHYL 4-DEOXY-α-D-*xylo*-HEXOPYRANOSID)URONATE*

Peak form	*δ (p.p.m.)*	*J (Hz)*	*Assignment*
Doublet	4.86	3.7 (1-2)	H-1
Quartet	4.34	12.5 (4a-5)	
		2.5 (4e-5)	H-5
Octet	3.92	9.7 (2-3)	
		11.4 (3-4a)	
		5.0 (3-4e)	H-3
Singlet	3.74		OAc
Quartet	3.47	3.7; 9.7	H-2
Singlet	3.40		OMe
Octet	2.32	5.0; 2.5	
		12.6 (4e-4a)	H-4*e*
Quartet	1.67	11.4; 12.5	
		12.6	H-4*a*

*Recorded in chloroform-*d* containing a trace of trichloroacetic acid.

reduction with lithium aluminum hydride into methyl 4-deoxy-α-D-*xylo*-hexopyranoside (**10**). The n.m.r. spectrum observed after addition of a trace of trifluoroacetic acid showed enough resolution to be analyzed by a first-order approach, although the multiplets of the two protons, that were formerly obscured by coupling with hydroxyl protons, were still partly overlapped by the two peaks originating from the methoxy protons. The peaks were assigned in analogy to the n.m.r. spectrum[1] of **5**, as indicated in Table I. The coupling constants indicate that compound **7** had, in chloroform solution, the *C1* conformation.

Fractionation of the products obtained after hydrogenation of **4** gave, in the first fraction, the minor component **6** which could not be crystallized. The main product **8** was obtained in crystalline form and characterized as a crystalline amide **15**. First-order analysis of the 100-MHz n.m.r. spectrum of compound **8** (Table II) suggested the structure to be methyl (methyl 4-deoxy-β-D-*xylo*-hexopyranosid)-uronate. Although the two methoxy peaks overlapped the multiplet formed by H-3, assignments could be made by comparison with the spectrum of the α anomer and by inspection of the observed coupling constants. These were consistent with a D-*xylo* structure adopting a *C1* conformation. Additional evidence was provided by lithium aluminum hydride reduction of compound **8**, which gave methyl 4-deoxy-β-D-*xylo*-hexopyranoside (**11**)[8,9].

TABLE II

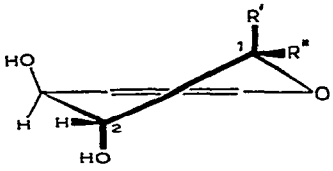
N.M.R. SPECTRAL DATA (100 MHz) OF METHYL (METHYL 4-DEOXY-β-D-*xylo*-HEXOPYRANOSID)URONATE*

Peak form	*δ (p.p.m.)*	*J (Hz)*	*Assignment*
Doublet	4.14	7.8 (1-2)	H-1
Quartet	4.08	12.2 (4a-5)	
		2.4 (4e-5)	H-5
Singlet	3.76		OAc
Multiplet	~3.7		H-3
Singlet	3.54		OMe
Quartet	3.31	7.8	
		8.8 (2-3)	H-2
Octet	2.29	5.2 (3-4e)	
		12.9 (4e-4a)	
		2.4	H-4*e*
Quartet	1.72	11.8 (3-4a)	
		12.9; 12.2	H-4*a*

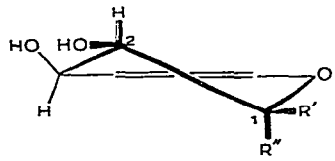
*Recorded in chloroform-*d* containing a trace of trichloroacetic acid.

Cook and Overend[2] have reported that the hydrogenation of **3** gave the L-*arabino* and D-*xylo* derivatives in a ratio of 4 to 5 when performed in ethanol solution (probably containing some water). The present results, obtained under essentially the same reaction conditions, show that the formation of the L-*arabino* compound is favored when the solvent is dry methanol. If the ratio of the two epimers is mainly determined by the accessibility of the double bond from either side of the

ring, the different yields may be explained in terms of different conformations adopted by the unsaturated uronic acid derivative in the two solvents. Since enopyranoses have been shown to form half-chair (H) conformations[10–12], compounds **3** and **4**



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may exist in either the H_2^1 (**16**) or the H_1^2 (**17**) form. Molecular models show that the "α"-side of **3** is more heavily shielded in the H_1^2 form by the bulky axial methoxy group than it is in the H_2^1 form, where this group is in equatorial position. Therefore, it seems that **3** adopts in ethanol the H_2^1 and in methanol the H_1^2 conformation. Steric hindrance by an axial methoxy group may account for the greater yield of the D-*xylo* epimer among the hydrogenation products of **4**, too. Inspection of the 100-MHz n.m.r. spectrum of this compound revealed that it adopted a H_2^1, rather than a H_1^2, conformation in chloroform solution. Two well separated doublets, each representing one proton, were observed at δ 6.19 (J 4.2 Hz) and δ 5.05 (J 3.6 Hz) and they were assigned to the protons H-4 and H-1, respectively. The small constant found for the coupling between H-1 and H-2 indicates their equatorial orientation and hence the H_2^1 conformation.

EXPERIMENTAL

General. — Melting points are corrected. Ether and tetrahydrofuran were purified by treatment with sodium and then distilled. Methanol was dried with magnesium and redistilled in presence of sodium immediately before use. T.l.c. was performed on Silica Gel G (Merck, Darmstadt, W. Germany) layers on normal plates or microscope slides with the following solvent systems: A, ethyl acetate–acetic acid–formic acid–water (18:4:1:3, v/v)[13]; B, ethyl acetate. The staining reagent was the mixture ethanol–conc. H_2SO_4–anisaldehyde (90:5:5, v/v)[14]. The g.l.c. data were obtained with the procedure described recently[15]; the R_E-values are the retention times of the per(trimethylsilyl) derivatives relative to the retention time of tetra-*O*-(trimethylsilyl)-*meso*-erythritol used as internal standard. Column chromatography was performed on silica gel (0.05-0.2 mm, Merck, Darmstadt, W. Germany); fractions of 120 drops (4–5 ml) were collected with an automatic fraction collector equipped with a drop counter at a flow rate of 0.4–0.6 ml/min. The fractionations were analysed by t.l.c. on microscope slides. Concentrations and evaporations were made in a flash evaporator at a bath temperature of 45–55° at 11 Tor. U.v. measurements were recorded with a Zeiss spectrophotometer, model PMQ II. N.m.r. spectra were recorded in chloroform-*d* with a Varian 100-MHz n.m.r. spectrometer; the internal standard was tetramethylsilane. The elementary analyses of samples dried for

10–15 h at room temperature at 0.05–0.1 Tor were performed in the microanalytical laboratory, Institute for Organic Chemistry, Swiss Federal Institute of Technology, Zurich. Methyl (methyl α-D-galactopyranosid)uronate (**1**), m.p. 147–148°, and methyl (methyl β-D-galactopyranosid)uronate (**2**), m.p. 191–192°, were isolated by fractional crystallization from a methyl glycoside methyl ester mixture obtained by boiling D-galacturonic acid in methanolic hydrochloric acid[13,16,17]. A reference sample of methyl 4-deoxy-α-D-*xylo*-hexopyranoside (**10**) was kindly provided by Prof. W. G. Overend, Birkbeck College, London, England.

*Methyl (methyl 4-deoxy-β-*L-threo-*hex-4-enopyranosid)uronate* (**3**). — The following details are given in addition to the procedure described previously[3]. A two-neck round bottom flask (100 ml) was equipped with a reflux condenser and with a separatory funnel having a second reflux condenser attached to the top of the funnel; both condensers were protected by a drying tube containing silica gel. Compound **1** was dried for 3 days at room temperature in presence of phosphoric anhydride at 0.05–0.1 Tor, and then placed into the flask. In the second distillation, methanol was received directly into the separatory funnel which had a glass-wool plug in its bottom tube. One half of the methanol was added to the flask which was then heated in a glycerol bath. To the other half remaining in the funnel, the appropriate amount of sodium was added. The sodium was peeled and weighed under dry petroleum ether (b.p. 70–90°), or added in ampoules prepared according to Schwarzenbach and Fischer[18]. After the sodium had reacted with the methanol, 0.5M magnesium methoxide (10 drops) was added. The methoxide solution was then filtered through the glass-wool plug into the boiling methanolic solution. The product was recovered as previously described[3], and was purified by adsorption chromatography on a silica gel column and elution with ethyl acetate (this method gave sharper separations and purer products) to give a colorless syrup[2,3]; g.l.c. data: R_E value 2.1 (SE-52).

For determinations of the optimal reaction time, samples (0.5 ml) were withdrawn with a syringe from the reaction mixture (containing 2.97 g of **1** in 40 ml of 1.5M sodium methoxide solution) at 5–10 min intervals. Each sample was neutralized with a slurry of dry[13] Dowex-50 (H^+) (0.5 g) in dry methanol (5 ml) in a 100-ml volumetric flasks which was filled to the mark with methanol. Samples (5 ml) of these solutions were transferred to another 100-ml volumetric flask and diluted with methanol. The absorbance of these solutions, measured at 235 nm, gave the following readings (min): .008 (1); .056 (5); .122 (15); .148 (35); .200 (40); .177 (50); .184 (55); .172 (60); .163 (65); .177 (70); .174 (75); .173 (80). The calibration curve of the u.v. absorption versus concentration of **3** is a straight line (1.15 mg per 100 ml gives an absorbance of 0.331).

T.l.c. data: R_F values in solvent systems A and B; compound **1**, 0.38–0.40 (A) and 0.00–0.06 (B); methyl α-D-galactopyranosiduronic acid, 0.24–0.26 (A) and 0.00 (B); compound **3**, 0.71–0.73 (A) and 0.34–0.36 (B); methyl 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid, 0.61–0.63 (A) and 0.00–0.09, tailing (B).

*Methyl (methyl 4-deoxy-α-*L-threo-*hex-4-enopyranosid)uronate* (**4**). — Methyl

(methyl 2,3-di-*O*-acetyl-4-deoxy-α-L-*threo*-hex-4-enopyranosid)uronate (1 g) was dissolved in 0.5M sodium methoxide (60 ml), and the solution was kept for 16 h at room temperature. The mixture was neutralized with dry Dowex 50 (H^+). After filtration, the resin was washed twice with methanol (50-ml portions). The combined methanolic solution was evaporated to dryness to give a slightly colored syrup. It was redissolved in dry methanol (5 ml), treated with a solution of diazomethane in ether to reesterify the saponified product, and again evaporated to dryness. The residue was dissolved in ethyl acetate and chromatographed (column: 2.3 cm × 21 cm). Fractions No.25-55 contained compound **4** (according to g.l.c.; R_E values: 2.2 on SE–52 and 2.0 on SE–30). After evaporation of the solvent, the combined fractions gave a colorless syrup, 510 mg (72%); n_D^{27} 1.4932; $[\alpha]_D^{27}$ −66.0° (*c* 1.166, abs. methanol). N.m.r. data: δ 6.19 (1-proton doublet, *J* 4.2 Hz, H-4); δ 5.05 (1-proton doublet, *J* 3.6 Hz, H-1); δ 4.08-3.82 (2-proton multiplet, H-2 and H-3); δ 3.80 (3-proton singlet, -OAc); δ 3.49 (3-proton singlet, -OMe).

Anal. Calc. for $C_8H_{12}O_6$: C, 47.06; H, 5.92; Found: C, 45.81; H, 5.92.

*Methyl (methyl 4-deoxy-β-*L-arabino-*hexopyranosid)uronate* (**5**) *and methyl (methyl 4-deoxy-α-*D-xylo-*hexopyranosid)uronate* (**7**). — A 0.25M solution of **3** in dry methanol was hydrogenated with 10% palladium-on-charcoal (1 mg per 0.02 mmole of **3**) at 22–24°. The mixture was stirred with a magnetic stirrer at a hydrogen pressure slightly higher than atmospheric pressure, and the stirring was continued for 1 h after the initial, quick hydrogen-uptake had ceased. The catalyst was filtered off, and washed with methanol. The combined methanolic solutions were evaporated to dryness to give a colorless syrup in nearly theoretical yield (97–98%). Fractionation on silica gel (column: 2.3 × 21 cm) of the hydrogenation product (1.5 g) gave product **5** in fractions No. 21–34. Concentration of the combined fractions gave a crystalline mass (800 mg, 52%). Recrystallization from hot ethyl acetate gave colorless square plates, m.p. 82–83°[1]; g.l.c. data R_E values: 1.8 (SE–52) and 1.4 (SE–30), and after saponification[15] 2.0 (SE–30). Fractions No. 35-55 (325 mg) contained a mixture and fractions No. 56–135 compound **7**. Evaporation of the solvent of the combined, latter fractions gave a colorless syrup, 168 mg (11%), which crystallized from hot, dry ether in long needles, m.p. 77.5–78.0°, $[\alpha]_D^{28}$ +123.3° (*c* 0.912, dry methanol). G.l.c. data: R_E values; 2.3 (SE–52) and 1.8 (SE–30), and after saponification[15] 2.7 (SE–30).

Anal. Calc. for $C_8H_{14}O_6$: C, 46.60; H, 6.84; Found: C, 46.52; H, 6.80.

*(Methyl 4-deoxy-β-*L-arabino-*hexopyranosid)uronamide* (**13**). — Compound **5** (100 mg) was dissolved in dry methanol (5 ml) and the solution was cooled with an ice-bath. A methanolic solution of ammonia (20 ml), saturated at 0°, was added. The mixture was kept for 24 h in a refrigerator, and then was evaporated to dryness. The residue was dissolved in dry methanol and the solution evaporated to dryness to remove completely the ammonia. Two crystallizations from boiling ethyl acetate gave colorless prisms, m.p. 126.5–127.0°, $[\alpha]_D^{28}$ +148.2° (*c* 0.880, dry methanol).

Anal. Calc. for $C_7H_{13}NO_5$: C, 43.97; H, 6.85; N, 7.33; Found: C, 43.83; H, 6.84; N, 7.37.

(*Methyl 4-deoxy-α-*D-xylo-*hexopyranosid*)*uronamide* (**14**). — This compound was prepared from **7** (130 mg) by a procedure identical to that for **13**. After two recrystallizations from methanol–ethyl acetate, the crude amide gave colorless prisms, m.p. 163.0–163.5°, $[\alpha]_D^{25}$ +112.9° (*c* 1.130, dry methanol).

Anal. Calc. for $C_7H_{13}NO_5$: C, 43.97; H, 6.85; N, 7.33; Found: C, 44.10; H, 7.01; N, 7.20.

*1,6-Anhydro-4-deoxy-*L-arabino-*hexopyranose* (**12**). — To a solution of **5** (720 mg) in dry tetrahydrofuran (5 ml) a suspension of lithium aluminum hydride (720 mg) in dry tetrahydrofuran (20 ml) was added dropwise at room temperature with stirring (magnetic stirrer). After 30 min, the addition was complete, the mixture was stirred for another 30 min, boiled for 1 h at reflux, and then cooled with an ice-bath. An equal volume of water was added, and the mixture was filtered. The precipitate was extracted twice with hot water. The combined filtrates were passed through a column of Dowex-50 (H^+) containing a 5-fold excess of resin. The column was washed with 3 volumes of water, and the eluate and washings were evaporated to dryness. The residue (570 mg) was fractionated (column: 2.3 cm × 21.5 cm) with ethyl acetate–methanol (9:1, v/v) to give methyl 4-deoxy-*β*-L-*arabino*-hexopyranoside (**9**) in the fractions No. 35-65. Evaporation of the solvent gave a colorless syrup (230 mg, 37%). G.l.c. data: R_E value; 1.4 (SE–30).

The methyl glycoside **9** was treated in boiling water with Dowex-50 (H^+) for 6 h. The resin was filtered off, and washed 3 times with water. The combined filtrate and washings were evaporated to dryness. The residual colorless syrup was fractionated (column: 1.8 cm × 8.0 cm) with acetone. Compound **12** was eluted in fractions No. 4-10. Evaporation of the combined fractions gave a crystalline mass (138 mg, 60%) which, on recrystallization from hot ethyl acetate, gave colorless prisms[1], m.p. 106–107°. G.l.c. data: R_E values; 1.00 (SE–52 and SE–30).

*Methyl 4-deoxy-α-*D-xylo-*hexopyranoside* (**10**). — Reduction of **7** (195 mg) was performed as described for compound **5**. The resulting syrup (156 mg) was purified by fractionation, as described for **9** to give a colorless syrup (131 mg, 76%), which crystallized from hot ethyl acetate as colorless prisms, m.p. and mixed m.p. 88–89°, $[\alpha]_D^{26}$ +166.9° (*c* 1.021, dry methanol). G.l.c. data: R_E value; 1.8 (SE–30). Hedgley *et al.*[9] reported m.p. 90°, $[\alpha]_D$ +168.2° (methanol); Cook and Overend[2], m.p. 89–90°, $[\alpha]_D$ +167.3° (methanol).

Methyl (*methyl 4-deoxy-β-*D-xylo-*hexopyranosid*)*uronate* (**8**). — The hydrogenation of **4** was performed as described for compound **3**. The resulting product (650 mg) was fractionated (column: 2.3 cm × 23.5 cm) to give a minor product in fractions No. 45-50. After evaporation, a syrup (20 mg, 3%) was obtained which could not be crystallized. This compound was assumed to be methyl (methyl 4-deoxy-α-L-*arabino*-hexopyranosid)uronate (**7**). G.l.c. data: R_E values; 1.8 (on SE–52 and on SE–30). Fractions No. 51–56 contained a mixture of both hydrogenation products (62 mg).

Compound **8** was eluted in fractions No. 57-120 which gave a crystalline mass after evaporation (451 mg, 68%). Recrystallization from hot ethyl acetate gave

colorless needles, m.p. 110.5–111.0° $[\alpha]_D^{23}$ −38.0° (*c* 1.396, dry methanol). G.l.c. data: R_E values; 2.2 (SE–52 and SE–30).

Anal. Calc. for $C_8H_{14}O_6$: C, 46.60; H, 6.84; Found: C, 46.69; H, 6.79.

(*Methyl 4-deoxy-β-*D-xylo-*hexopyranosid*)*uronamide* (**15**). — This product was prepared from **8** as described for **13**, and it was recrystallized twice from boiling ethyl acetate to give fine needles, m.p. 194.5–195.0°, $[\alpha]_D^{26}$ −112.3° (*c* 1.172, dry methanol).

Anal. Calc. for $C_7H_{13}NO_5$: C, 43.97; H, 6.85; N, 7.33; Found: C, 43.99; H, 6.97; N, 7.23.

*Methyl 4-deoxy-β-*D-xylo-*hexopyranoside* (**11**). — Reduction of **8** (200 mg), as described for **12**, gave a crystalline product (160 mg, 91%) which was recrystallized 3 times from hot ethyl acetate into colorless needles, m.p. 146–147°, $[\alpha]_D^{25}$ −35.6° (*c* 0.917, water). G.l.c. data: R_E values; 2.2 (SE–52) and 2.1 (SE–30). Dahlgard *et al.* [8] reported m.p. 143°, $[\alpha]_D^{25}$ −35.5° (water); Hedgley *et al.* [9], m.p. 145–146°, $[\alpha]_D$ −35.7° (*c* 0.8, water).

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METHYL TERMINAL-4-DEOXY-, TERMINAL-4-*O*-ETHYL-, AND TERMINAL-4-*O*-BUTYL-MALTO-OLIGOSACCHARIDES: SYNTHESIS, AND HYDROLYSIS WITH *beta*-AMYLASE

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ABSTRACT

Reactions of perbenzylated methyl terminal-4-hydroxymalto-oligosaccharides (**1**) provided routes to the title compounds. Alkylation followed by debenzylation yielded the terminal-4-*O*-ethyl and terminal-4-*O*-butyl derivatives. The terminal-4-deoxy compounds were formed from **1** by sulfonylation, displacement of the sulfonyloxy group with thiocyanate, and hydrogenolysis in the presence of Raney nickel. *beta*-Amylase acted on the mixture of methyl terminal-4-deoxymalto-oligosaccharides to convert it into 4-deoxymaltose, maltose, methyl maltoside, and methyl D-glucopyranoside.

INTRODUCTION

We have previously reported[1] the synthesis of methyl terminal-4-*O*-methyl-malto-oligosaccharides, and we suggested that the method used should be a general one by which other terminally substituted oligosaccharides could be prepared. This method has now been used for synthesizing methyl terminal-4-*O*-ethyl-, -4-*O*-butyl-, and -4-deoxy-malto-oligosaccharides. Synthesis of all four compounds proceeded *via* the perbenzylated methyl terminal-4-hydroxymalto-oligosaccharides. The alkyl ethers were then prepared by alkylation of the one free hydroxyl group, followed by debenzylation.

Methyl terminal-4-deoxymalto-oligosaccharides were synthesized by a nucleophilic displacement of the sulfonyloxy group of perbenzylated methyl terminal-4-*O*-sulfonylmalto-oligosaccharides with potassium thiocyanate. The 4-*O*-(thiocyano) group in the axial position (D-*galacto* configuration) was removed, together with the benzyl ether protecting groups, by Raney nickel-catalyzed hydrogenolysis.

Sulfonates have frequently been used in synthetic carbohydrate chemistry through reaction with various nucleophiles in S_N2 reactions[2,3]. However, only one of the syntheses reported used the benzyl group for protection in such nucleophilic displacements[4], and the conversion of lactose into cellobiose seems to be the only previous instance of application of this type of reaction to an oligosaccharide[5].

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DISCUSSION

Two additional series of methyl terminal-4-*O*-alkylmalto-oligosaccharides, namely, methyl terminal-4-*O*-ethyl- and -4-*O*-butyl-malto-oligosaccharides, have now been synthesized by the method previously described[1]. In both cases, a homologous series of oligosaccharides was obtained, as shown by t.l.c. (see Tables I and II).

TABLE I

R_F VALUES[a] FOR T.L.C. OF THE PERBENZYLATED METHYL TERMINAL-4-*O*-SUBSTITUTED-MALTO-OLIGOSACCHARIDES

Degree of polymerization	*4-Hydroxy*		*4-O-Methyl*		*4-O-Ethyl*		*4-O-Butyl*	
	α	β	α	β	α	β	α	β
1	0.31	0.36	0.47	0.52	0.54	0.57	0.58	0.63
2	0.22	0.25	0.41	0.44	0.46	0.49	0.48	0.52
3	0.16	0.18	0.33	0.37	0.39	0.42	0.41	0.45
4	0.10	0.12	0.25	0.29	0.30	0.34	0.33	0.37
5	0.06	0.09	0.18	0.22	0.23	0.26	0.26	0.29
6	—	—	—	—	0.17	0.20	0.19	0.22

[a]Silica Gel H, Brinkmann Instruments Co. Irrigant: 5:1 (v/v) petroleum ether (b.p. 30-60°)–acetone[9].

TABLE II

R_F VALUES[a] FOR T.L.C. OF THE METHYL TERMINAL-4-SUBSTITUTED-MALTO-OLIGOSACCHARIDES

Degree of polymerization	*4-Hydroxy*	*4-O-Methyl*	*4-O-Ethyl*	*4-O-Butyl*	*4-Deoxy*
1	0.49	0.54	0.63	0.74	0.54
2	0.31	0.35	0.43	0.52	0.35
3	0.16	0.21	0.25	0.34	0.20
4	0.08	0.12	0.14	0.19	0.09
5	0.04	0.06	0.08	0.10	0.05
6	0.00	0.01	0.04	0.06	—

[a]Silica Gel H, Brinkmann Instruments Co. Irrigant: 9:6:3:1 (v/v) butyl alcohol–acetic acid–ether–water[10].

Characterization was accomplished by acid-catalyzed hydrolysis, followed by analysis of the product by t.l.c. (see Table III) and by g.l.c. of their per(trimethylsilyl) derivatives[6] (see Table IV). The results of this analysis showed that, as the size of the alkyl group is increased, there is a greater tendency toward dealkylation during hydrogenolytic debenzylation, perhaps because of the low solubility of such compounds in absolute ethanol. When an excess[1] of Raney nickel is employed, the compounds are bound to it, thus giving the catalyst an opportunity to remove some of the butyl groups. When methyl 2,3,6-tri-*O*-benzyl-4-*O*-butyl-D-glucopyranoside is treated under the same conditions, no debutylation is observed[7].

TABLE III

R_F VALUES[a] FOR T.L.C. OF THE ACID-HYDROLYSIS PRODUCTS OF THE METHYL TERMINAL-4-SUBSTITUTED-MALTO-OLIGOSACCHARIDES

Acid-hydrolysis products	R_F
D-Glucose	0.41
4-*O*-Methyl-D-glucose	0.47
4-*O*-Ethyl-D-glucose	0.61
4-*O*-Butyl-D-glucose	0.71
4-Deoxy-D-*xylo*-hexose	0.50

[a]Silica Gel H, Brinkmann Instruments Co. Irrigant: 9:6:3:1 (v/v) butyl alcohol–acetic acid–ether–water[10].

TABLE IV

G.L.C. DATA FOR THE PER(TRIMETHYLSILYL)ATED PRODUCTS FROM THE ACID HYDROLYSIS OF THE METHYL TERMINAL-4-SUBSTITUTED-MALTO-OLIGOSACCHARIDES

TMS derivative of	*Retention time (min)* *Non reducing end-unit*		α-D-*Glucose*	β-D-*Glucose*
	α *Anomer*	β *Anomer*		
Malto-oligosaccharides				
4-*O*-methyl-	9.4	15.4	11.8	19.2
4-*O*-ethyl-	7.9	14.0	11.5	19.0
4-*O*-butyl-	14.4	25.0	11.3	18.5
4-deoxy-	5.1	7.2	12.1	18.5
Standards				
D-Glucose			11.8	19.5
4-*O*-methyl-	9.5	15.4		
4-*O*-ethyl-	7.9	14.1		
4-*O*-butyl-	14.4	25.0		
4-deoxy-D-*xylo*-hexose	5.1	7.2		

Methyl terminal-4-deoxymalto-oligosaccharides were prepared by *p*-toluenesulfonylation or methanesulfonylation of the same perbenzylated methyl terminal-4-hydroxymalto-oligosaccharides obtained from the methanolysis of tri-*O*-benzylamylose[1]. Treatment of the perbenzylated methyl terminal-4-*O*-sulfonylmalto-oligosaccharides (**6** or **7**) with an excess of potassium thiocyanate in *N*,*N*-dimethylformamide at 140° gave a 50% yield of the terminal 4-deoxy-4-(thiocyano) derivative. In this product, the configuration at the carbon atom bearing the thiocyano group is inverted (terminal-D-*galacto* configuration) in comparison with **6** and **7**. Raney nickel-catalyzed hydrogenolysis[8] of this product gave a series of methyl terminal-4-deoxymalto-oligosaccharides contaminated with an approximately equimolar amount of the methyl terminal-4-hydroxymalto-oligosaccharides. No attempts have yet been made to separate these two series of malto-oligosaccharides. The series of reactions

used in preparing these deoxy oligosaccharides were first effected on several simple sugar sulfonates, and similar yields were observed for each[7]. The compounds used were methyl 2,3,6-tri-*O*-benzyl-4-*O*-(methylsulfonyl)-D-glucopyranoside, methyl 2,3-di-*O*-benzyl-4,6-di-*O*-(methylsulfonyl)-(and *p*-tolylsulfonyl)-α-D-glucopyranoside, methyl 2,3-di-*O*-benzoyl-4-*O*-*p*-tolylsulfonyl-6-*O*-trityl-α-D-glucopyranoside, methyl 2,3-di-*O*-benzoyl-4-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside, methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(methylsulfonyl)-(and *p*-tolylsulfonyl)-α-D-glucopyranoside, methyl 4-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside, methyl 2,3,4-tri-*O*-benzoyl-6-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside, and methyl 2,3-di-*O*-benzoyl-4,6-di-*O*-(methylsulfonyl)-(and *p*-tolylsulfonyl)-α-D-glucopyranoside.

Characterization of the series of terminal-4-deoxyoligosaccharides was accomplished by acid-catalyzed hydrolysis followed by analysis by t.l.c. (see Table III) and by g.l.c.[6] (see Table IV), with 4-deoxy-D-*xylo*-hexose as the standard.

The synthesis of these methyl terminal-4-substituted malto-oligosaccharides was undertaken in order to provide compounds to be used for ascertaining the mechanism of *beta*-amylase action.

Methyl terminal-4-hydroxy-, -4-*O*-methyl-, and -4-deoxy-malto-oligosaccharides were treated with *beta*-amylase in an acetate buffer (pH 4.8) for 24 h at 25°. The products were analyzed by t.l.c. (see Table V), with the following results. Methyl

TABLE V

R_F VALUES[a] FOR T.L.C. OF THE PRODUCTS FROM *beta*-AMYLASE HYDROLYSIS

Product from hydrolysis with beta-*amylase*	R_F
Methyl D-glucopyranoside	0.49
Maltose	0.26
Methyl maltoside	0.31
4-Deoxymaltose	0.32

[a]Silica Gel H, Brinkmann Instruments Co. Irrigant: 9:6:3:1 (v/v) butyl alcohol–acetic acid–ether–water[10].

terminal-4-hydroxymalto-oligosaccharides were completely converted into maltose, methyl D-glucopyranoside [from the component having degree of polymerization (D.P.) 1 and from odd-numbered oligosaccharide chains] and methyl maltoside (from the reducing end). No methyl maltotrioside was found, even though Peat *et al.*[11] have reported that no hydrolysis of methyl maltotrioside occurs with low concentrations of *beta*-amylase. Methyl terminal-4-*O*-methylmalto-oligosaccharides[1] showed no reaction with *beta*-amylase. A trace amount of methyl terminal-4-hydroxymalto-oligosaccharides in this preparation (from the oligosaccharides obtained from the nonreducing end of tri-*O*-benzylamylose, which would have a benzyl group on O-4 before methylation) was converted into maltose. Therefore, this reaction could be used to remove any nonderivatized methyl terminal-4-hydroxymalto-oligosaccharides from any methyl 4-*O*-alkyloligosaccharides.

Methyl terminal-4-deoxymalto-oligosaccharides were converted into maltose and a faster-moving component (t.l.c.), which has been designated terminal-4-deoxymaltose. From these results, it may tentatively be concluded that the 4-hydroxyl group is not a necessary binding site for the enzyme. However, the present article deals primarily with the synthesis of these compounds; the enzymic data are merely preliminary, and this work will be followed by a more detailed investigation.

EXPERIMENTAL

Gas–liquid chromatography. — This was performed with a Varian Aerograph Model 204-B gas chromatograph with a flame-ionization detector. Per(trimethylsilyl) (TMS) ethers were prepared by the method of Brobst and Lott[6], and injected into a stainless-steel column (6-ft by 0.25-in i.d.) packed with 5% SE-30 silicone gum rubber (methyl) on acid-washed, 60–80 mesh, dichlorodimethylsilane-treated Chromosorb W. The column temperature was 160°, and the temperature of the injection port and detector was 300°. The carrier gas was helium, flowing at the rate of 35 ml/min at an inlet pressure of 40 lb. in^{-2}. Hydrogen and air were supplied to the detector at the rate of 15 ml/min, at an inlet pressure of 5 lb. in^{-2}, and at 300 ml/min at an inlet pressure of 3 lb. in^{-2}, respectively.

Perbenzylated methyl terminal-4-O-ethylmalto-oligosaccharides (2). — The mixture of perbenzylated methyl terminal-4-hydroxymalto-oligosaccharides[1] (1) (2.05 g) obtained from a 24-h methanolysis of tri-*O*-benzylamylose[1] was dissolved in 30 ml of *N*,*N*-dimethylformamide in a 100-ml, round-bottomed flask. Sodium hydride (3.0 g) was added, and the reaction was allowed to proceed for 1 h at room temperature; then, 20 ml of ethyl bromide was added, and the mixture was stirred overnight, and processed as previously described[1]. The yield was 2.09 g (100%) of a pale-yellow syrup. The reaction was shown by t.l.c. to be complete (see Table I), and the i.r. spectrum of the syrup showed no hydroxyl absorption at 3600 cm^{-1}.

Methyl terminal-4-O-ethylmalto-oligosaccharides (3). — A solution of **2** (1.7 g) in 50 ml of chloroform was placed in a hydrogenation flask; the chloroform was removed under diminished pressure, absolute ethanol (300 ml) was added, and the mixture was heated until refluxing. Freshly activated Raney nickel (5 g) was carefully added to the hot solution, and the insoluble material was scraped off the inside of the flask, so that it could be adsorbed onto the catalyst. The flask was then attached to a Parr hydrogenator, and flushed three times with hydrogen. Hydrogenolysis was conducted for 48 h at 60°, under 65 lb. in^{-2} of hydrogen. The Raney nickel was filtered off, and the filtrate was evaporated to dryness under diminished pressure. Partially benzylated material was removed from the resulting syrup by extraction with chloroform, and the syrup remaining (0.242 g) was analyzed by t.l.c. (see Table II). The catalyst was then washed with chloroform (100 ml) to remove any benzylated compounds. After filtration, the Raney nickel was stirred with water (200 ml) to remove oligosaccharides of higher molecular weight. The Raney nickel was filtered off, and the filtrate was stirred with a mixed, anion-cation exchange resin.

Evaporation gave 0.206 g of compounds having a D.P. of 6–16. The total yield of completely debenzylated malto-oligosaccharides was 0.488 g (65%).

Perbenzylated methyl terminal-4-O-butylmalto-oligosaccharides (**4**). — These oligosaccharides were prepared by the procedure used for preparing the 4-*O*-ethyl derivative, except that 1-bromobutane (40 ml) was used instead of ethyl bromide; yield 2.14 g (100%). The syrup was analyzed by t.l.c. (see Table I), and its i.r. spectrum showed no hydroxyl absorption at 3600 cm^{-1}.

Methyl terminal-4-O-butylmalto-oligosaccharides (**5**). — Mixture **4** (1.25 g) was debenzylated under the conditions used for the 4-*O*-ethyl derivative; yield 0.289 g (56%). The product was analyzed by t.l.c. (see Table II).

Perbenzylated methyl terminal-4-O-p-tolylsulfonylmalto-oligosaccharides (**6**). — The perbenzylated methyl terminal-4-hydroxymalto-oligosaccharides[1] (2.05 g), obtained from a 24-h methanolysis of tri-*O*-benzylamylose, were dissolved in anhydrous pyridine (25 ml), *p*-toluenesulfonyl chloride (2.0 g) was added, and the mixture was stirred for 18 h at room temperature and then for 18 h at 70°. The mixture was cooled to room temperature, diluted with chloroform, and successively washed with water (several times), 0.5M sulfuric acid, dilute sodium hydrogen carbonate solution, and water. Evaporation of the chloroform gave a syrup weighing 2.1 g (92%). I.r. analysis showed no hydroxyl absorption at 3600 cm^{-1}, but strong bands at 1350–1330 ($-SO_2$ asymmetrical stretch), 1140 ($-SO_2$ symmetrical stretch), and 810 cm^{-1} (out-of-plane C–H deformation due to a 1,4-substituted benzene ring).

Perbenzylated methyl terminal-4-O-(methylsulfonyl)malto-oligosaccharides (**7**). — A 24-h methanolysis syrup (1.0 g) from tri-*O*-benzylamylose was dissolved in anhydrous pyridine (40 ml), methanesulfonyl chloride (5 ml) was added, and the reaction was stirred for 24 h at room temperature. The mixture was processed as described for **6**; yield 1.05 g (95%). I.r. analysis showed no hydroxyl absorption at 3600 cm^{-1}, but strong sulfonyl absorptions at 1350 and 1140 cm^{-1}.

Perbenzylated methyl terminal-4-deoxy-4-(thiocyano)malto-oligosaccharides (**8**). — To solution of **6** (1.0 g) in 40 ml of *N,N*-dimethylformamide was added potassium thiocyanate (1.0 g), and the mixture was stirred for 24 h at 140°. The solvent was evaporated off under diminished pressure, and the resulting syrup was washed several times with water (to remove final traces of solvent); yield 0.9 g. I.r. analysis of the syrup showed the presence of a strong absorbance at 2200 cm^{-1} due to thiocyanate, but small bands for sulfonate were still present. Similar results were obtained when the methanesulfonate (**7**) was treated under the same conditions.

Methyl terminal-4-deoxymalto-oligosaccharides (**9**). — Mixture **8** (0.9 g) was treated under the debenzylation conditions described for the perbenzylated methyl terminal-4-*O*-alkylmalto-oligosaccharides. The syrup obtained (0.336 g, 100%) was analyzed by t.l.c. (see Table II). Several components that corresponded to the methyl terminal-4-hydroxymalto-oligosaccharides were present.

Acid-catalyzed hydrolysis. — The methyl terminal-4-*O*-ethyl- (**3**), -4-*O*-butyl- (**5**), and -4-deoxy-malto-oligosaccharides (**9**) (50 mg) were each hydrolyzed in 0.5M sulfuric acid (30 ml) for 36 h at 80°. The respective solutions were then neutralized

with Amberlite IR-45 (OH^-) anion-exchange resin (Rohm and Haas Co.), and evaporated to dryness under diminished pressure. The hydrolyzates were analyzed by t.l.c. (see Table III) and g.l.c. (see Table IV).

Hydrolysis with beta-*amylase.* — *beta*-Amylase (5 mg, 3750 units; Worthington Biochemical Corp., Freehold, N. J.) was placed on a column (20-ml) of coarse Sephadex G-25 (Pharmacia Fine Chemicals Inc., New Market, N.J.) pre-equilibrated with 100 mM acetate buffer (pH 4.8). Fractions (1.4 ml) were collected, and analyzed in a Gilford Spectrophotometer (Model 2000) at 280 nm. The seventh through ninth fractions contained the enzyme, and were combined and diluted up to a volume of 10 ml (3.29 μM).

The methyl terminal-4-hydroxy- (**1**), -4-*O*-methyl-[1], and -4-deoxy-malto-oligosaccharides (**9**) (15 mg) were each dissolved in 1 ml of 100 mM acetate buffer (pH 4.8) in separate flasks. To each flask, 1.0 ml of *beta*-amylase solution (3.29 μM) was added, and reaction was allowed to proceed for 24 h at 25°. The solutions were analyzed by t.l.c. (see Table V).

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STRUCTURE OF THE DEXTRAN OF THE TIBI GRAIN

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ABSTRACT

The dextran ($[\alpha]_D$ +206° in M NaOH) of the Tibi grain has been shown by partial acid hydrolysis, methylation, and periodate oxidation studies to be mainly an (1→6)-α-D-glucan, having branches at C-3. Partial acid hydrolysis gave isomaltose. Hydrolysis of the methylated dextran (1 mole) gave 2,3,4,6-tetra- (1 mole), 2,3,4-tri- (17.1 moles) and 2,4-di-*O*-methyl-D-glucoses (1.1 moles), and a small proportion of 2,4,6-tri-*O*-methyl-D-glucose which indicated the presence of a small proportion of (1→3)-linked residues in the molecule. A small proportion of 1,6-anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose (1.2 moles) was also isolated. Periodate oxidation of the dextran (DP 3630), followed by reduction and mild acid hydrolysis gave glycerol (56 moles), erythritol (1 mole), and 1-*O*-α-D-glucopyranosylglycerol (6 moles). Glucosylglycerol derives from the linear and branched sequences, G*p*-(1→3)-G*p*-(1→6)-G*p*-(1→3 and →6)-G*p*-(1→6)-. The structure of the dextran of the Tibi grain

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↑
1

is discussed on the basis of these findings.

INTRODUCTION

Tibi is an acidic, mildly alcoholic drink prepared by fermentation of a sucrose solution by Tibi grains. Figs, dates, raisins, or ginger roots provide the growth factors. The grains are a symbiotic association of *Lactobacillus brevis*, *Streptococcus lactis*, and *Saccharomyces cerevisiae*[1]. After one or two days[1] fermentation at room temperature the grains are separated and can be used again by immersing them in a fresh medium. The process can be repeated *ad infinitum*. Asepsis is not observed during the fermentation.

The Tibi grains are translucent, waxy, and tough in consistency. Some believe that this fermentation is similar to the "ginger beer plant" used in England[2-4]. The Tibi grains have been described under various names[5] and should be distinguished from Kefir grains which acidify and coagulate milks[2]. The Tibi grains or "Tibi complex" originate from Mexico where they occur in the form of hard granules on the leaves of the Opuntia plant[6].

In 1939, Daker and Stacey[7, 8] studied an insoluble dextran ($[\alpha]_D$ +180°) produced by an organism (*Lactobacillus brevis*) isolated from the "Tibi complex". They found that the dextran contained mainly α-D-(1→6)-linked glucopyranosyl residues ($\overline{CL}$ 25, DP 500).

The present report deals with the structure of the dextran produced by the "Tibi complex". The "Tibi complex" produces a single insoluble polysaccharide containing only D-glucose. This dextran {$[\alpha]_D^{25}$ +206° *c* 0.36, M sodium hydroxyde)} was obtained free of cells and debris by alkaline dissolution, and precipitated by neutralisation. Attempts to fractionate the alkaline solution of the dextran by ethanol precipitation failed.

The dextran was methylated by the Haworth and the liquid ammonia procedures to give a methylated product {$[\alpha]_D^{25}$ +216° (chloroform); OMe, 45.6%}. On acid hydrolysis of this product, 2,4-di-, 2,3,4-tri-, 2,3,4,6-tetra-*O*-methyl-D-glucose and 1,6-anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose were isolated in the molar ratio 1.1:17.1:1.0:1.2. As shown by paper chromatography, the 2,3,4-tri-*O*-methyl-D-glucose fraction contained a small amount of 2,4,6-tri-*O*-methyl-D-glucose. Because of overlapping, the presence of trace amounts of 2,3,6-tri-*O*-methyl-D-glucopyranose could not be detected by paper chromatography, although erythritol was detected in the hydrolysate of the dextran degraded by the Smith procedure. The methylated sugars were identified by thin-layer chromatography and by comparison of the physical properties of derivatives. The presence of 1,6-anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose is a known artefact[9]. The structural features indicated by the isolation of these methylated glucoses are shown in Table I. The dextran is thus composed mainly of (1→6)-linked D-glucopyranosyl residues (18.3 moles) having branches at O-3 (1.1 moles). Equimolar quantities of 2,3,4,6-tetra- and 2,4-di-*O*-methyl-D-glucose were isolated in proportions that indicated a branched structure having a $\overline{CL}$ 18.

TABLE I

HYDROLYSIS OF THE METHYLATED TIBI DEXTRAN

Product of cleavage	*Mode of linkage*	*Molar proportions*
O-Methyl-D-glucose		
2,3,4,6-Tetra-	G*p*-(1→	1.0
2,3,4-Tri-	→6)-G*p*-(1→	17.1
2,4,6-Tri-	→3)-G*p*-(1→	not determined
2,4-Di-	→6)-G*p*-(1→ 3 ↑	1.1
1,6-Anhydro-2,3,4-tri-*O*-methyl-β-D-glucose	→6)-G*p*-(1→	1.2

The presence of (1→6)-, (1→4)-, (1→3)-, or (1→3, 1→6)-linked D-glucose residues was attested by the detection of glycerol (56 moles), erythritol (1 mole),

and glucosylglycerol (6 moles) in the hydrolyzate of the dextran degraded by the Smith procedure. No free D-glucose was detected. It should be noted that erythritol could result from either (1→4) linkages or incomplete oxidation of (1→6)-linked glucose residues. Prolonged periodate oxidation (10 days), however, afforded the same amount of erythritol. These data agree with the periodate oxidation data which indicate a molar ratio of (1→6)- to (1→3)- and (1→3, 1→6)-linked D-glucose residues of 9 to 1. It has been shown that, when a polyalcohol is hydrolyzed under mild conditions, the residues derived from adjacent, (1→6)-linked units would be sensitive to acid and give rise to glycerol, whereas, if a (1→3)- or (1→3, 1→6)-linked unit is flanked by (1→6)-linked units, mild acid hydrolysis would produce 1-*O*-D-glucosylglycerol[10,11].

From the proportion of 2,4-di- or 2,3,4,6-tetra-*O*-methyl-D-glucose and glucosylglycerol isolated, it was calculated that Tibi dextran contains approximately an equal proportion of (1→3)- and (1→3, 1→6)-linked glucose units.

When the product of reduction of Tibi dextran with sodium borohydride was oxidized with periodate, it produced an amount of formaldehyde corresponding to a DP of 3630 (assuming that the terminal reducing group is linked through O-6) or a DP of 7260 (assuming that the terminal reducing group was linked through O-3). A modified ferrocyanide method gave a DP of 3430 for the dextran, thus indicating that the terminal reducing group is more likely to be linked through O-6.

The presence of α-D-glucosidic bonds in the Tibi dextran is suggested by the high positive optical rotation of the dextran ($[\alpha]_D^{25}$ +206°), by the isolation of isomaltose from the partial acid hydrolyzate of the dextran and by the isolation of 1-*O*-α-D-glucosylglycerol in the mild acid hydrolyzate of dextran degraded by the Smith procedure.

The dextran {$[\alpha]_D^{25}$ +206° (M sodium hydroxide), DP 3630, $\overline{CL}$ 18} of the Tibi grain is probably similar to the dextran isolated by Daker and Stacey[7,8] {$[\alpha]_D$ +180° (water), DP 500, $\overline{CL}$ 25}. The discrepancies in the physical constants may be explained by the fact that the dextran was made water soluble by acid treatment, prior to methylation studies.

EXPERIMENTAL

General. — Paper chromatography was performed on Whatman No. 1 or No. 3 paper by the descending technique with the following solvent systems: (A) butyl alcohol–ethanol–water (3:2:1)[12]; (B) pyridine–ethyl acetate–water (2:5:7, upper phase)[13]; (C) 2-butanone–water azeotrope[14]; (D) butyl alcohol–ethanol–water–conc. ammonium hydroxide (40:60:49:1, upper phase)[15]; (E) benzene–ethanol–water–acetic acid (200:47:15:1, upper phase)[16]. Sugars were detected on paper with the ammoniacal silver nitrate reagent[17] or with a *p*-aminobenzoic acid–phosphoric acid mixture[18]. Silica gel chromatograms were revealed by iodine vapor or by spraying with M sulfuric acid and heating at 120°. R_{TMG} refers to the mobility relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose as unity. Reducing sugars were quantita-

tively determined by the phenol–sulfuric acid method[19] or by a modified Park and Johnson method[20]. Melting points were determined on a Büchi melting-point apparatus, and are uncorrected. Optical rotations were measured with a Zeiss automatic polarimeter, type LEP. All evaporations were effected *in vacuo* at 30–40° (bath temperature).

Tibi culture. — Tibi grains* (40 g) were inoculated in tap water (2 l) containing sucrose (63 g), two dry figs, and a quartered lemon. After one day at room temperature in an open jar, the medium was renewed, and the Tibi grains (40 g) were re-inoculated. About 30 g of grains had to be discarded daily. Such a stock culture has been kept in good condition for over two years, in our laboratory. Larger grains were obtained when the sucrose concentration was increased four-fold.

Isolation of Tibi dextran. — Fresh Tibi grains (1000 g) were thoroughly washed with distilled water in the cold until the supernatant was free of sugars (phenol–sulfuric acid method[19]). The grains were dissolved in M sodium hydroxide (2 l). The solution was clarified by centrifugation, neutralized with acetic acid, and dialyzed in the cold. The insoluble dextran was centrifuged, washed with ethanol and acetone, and dried *in vacuo*; yield 180 g, $[\alpha]_D^{25}$ +206° (*c* 0.36, M NaOH). The supernatant was free of soluble polysaccharides, as shown by addition of ethanol which did not cause any precipitation.

Properties of Tibi dextran. — Tibi dextran (500 mg) was heated for 15 h in 0.5M sulfuric acid (20 ml) at 100°. The hydrolyzate was cooled, neutralized with barium carbonate, and filtered. The filtrate was concentrated to a small volume. Paper chromatography (solvents A and B) showed the presence of glucose only. The glucose content of the dextran was determined by the phenol–sulfuric acid method[19] after the dextran had been hydrolyzed under the same conditions: 500 mg of dextran gave 542 mg (as D-glucose).

The Tibi dextran (1 g) was reduced with sodium borohydride and oxidized with periodate[20]. The liberated formaldehyde was determined with the chromotropic acid reagent[22]. The results indicate that 24.5 μmoles of formaldehyde were produced per mole of glucose residue. Thus the reduced Tibi dextran has a DP of 3630 (assuming that the reducing end-group is linked through O-6). A modified Park and Johnson reducing group-determination[22] showed that the Tibi dextran had DP 3430.

Periodate degradation of the Tibi dextran. — The dextran (1 g) was oxidized with 0.1M sodium periodate (100 ml) at 5° in the dark. At suitable intervals, aliquots were taken and the periodate consumption was determined by the arsenite method[23]; formic acid was determined by potentiometric titration with 10mM sodium hydroxide. The oxidation was complete after 70 h. The periodate consumption was 1.79 moles, and the formic acid production was 0.88 mole per anhydrohexose unit. The excess of periodate was decomposed by addition of ethylene glycol, and the solution was dialyzed at 0° against distilled water. The polyaldehyde was reduced with sodium borohydride according to the procedure of Smith and associates[24]. The polyalcohol

*Obtained through the courtesy of Prof. A. Camus (Institut National d'Agronomie, Paris).

was dialyzed against distilled water at 0° and hydrolyzed in 0.05M sulfuric acid for 18 h at 25°. The hydrolyzate was neutralized with barium carbonate, deionized, and evaporated to dryness. Paper chromatography (solvents A and B) revealed glycerol, erythritol, and glucosylglycerol which were separated by thick-paper chromatography (solvent A). Glucosylglycerol (11.1 mg) was determined by the phenol–sulfuric acid method[19], glycerol (202 mg) and erythritol (4.3 mg) by the chromotropic acid method at 570 nm[25]. The results showed that the molar ratio of glycerol, erythritol, and glucosylglycerol was 56:1:6.

Identification of 1-O-α-D-*glucopyranosylglycerol.* — Glucosylglycerol showed $[\alpha]_D^{25}$ +128° (*c* 0.24, water); [lit.[26] for 1-*O*-α-D-glucosylglycerol: $[\alpha]_D^{27}$ +128° (*c* 0.69, water)]. When glucosylglycerol was hydrolyzed with Amberlite IR-120 (H^+) for 6 h at 100°, it liberated glucose (0.95 mole) and glycerol (1 mole) which were separated by paper chromatography (solvent A) and determined by the phenol–sulfuric acid method[19] and the chromotropic acid reagent[25]. When glucosylglycerol was oxidized with 0.1M sodium periodate for 24 h, at 5° in the dark, 0.94 mole of formaldehyde was liberated per mole of glucosylglycerol, as determined by the chromotropic acid method[25].

Methylation of Tibi dextran. — Tibi dextran (5 g) was methylated twice by the Haworth method[27], and once by a modified Haworth method[28] in tetrahydrofuran; yield: 4.6 g; found: OMe 30.1%. Partially methylated dextran (4.2 g) was further methylated twice in liquid ammonia with sodium and methyl iodide[29]; yield: 2.7 g, $[\alpha]_D^{25}$ +216° (*c* 0.4, chloroform). The methylated dextran showed no absorption at 3400 cm^{-1} in the i.r. spectrum, thus indicating that the dextran was completely methylated.

Anal. Calc. for permethylated derivative: OMe 45.6. Found: OMe, 45.6.

Hydrolysis of the methylated Tibi dextran and identification of the cleavage products. — *A.* The methylated dextran (0.5 g) was hydrolyzed by the formic acid–dilute sulfuric acid method[30]. The hydrolyzate was neutralized by addition of barium carbonate, and concentrated to a small volume. Examination of the syrup by paper chromatography (solvents C and D) showed three components corresponding to 2,3,4,6-tetra-, 2,3,4-tri-, and 2,4-di-*O*-methyl-D-glucoses. The presence of 1,6-anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose (R_{TMG} 1.55) was detected by t.l.c. (solvent E), in addition to 2,3,4,6-tetra- (R_{TMG} 1.0), tri-*O*-methyl- (R_{TMG} 0.55) and di-*O*-methyl-glucoses (R_{TMG} 0.1). The different bands were separated by preparative t.l.c. (solvent E), revealed by iodine vapor, and eluted with ethanol.

*B. 1,6-Anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose.* — The fraction having R_{TMG} 1.55 (25.5 mg) showed $[\alpha]_D^{25}$ +6° (*c* 0.5, methanol); [lit.[31]: $[\alpha]_D$ +10° (*c* 1.5, methanol)]. On t.l.c. in solvent E, a single component having a mobility identical with that of synthetic 1,6-anhydro-2,3,4-tri-*O*-methyl-β-D-glucopyranose was detected. Upon acid hydrolysis in M sulfuric acid (7 h, 100°), 2,3,4-tri-*O*-methyl-D-glucopyranose was detected by paper chromatography (solvents C and D).

*C. 2,3,4,6-Tetra-*O*-methyl-D-glucopyranose.* — The fraction having R_{TMG} 1.0 (25.1 mg) showed $[\alpha]_D^{25}$ +81° (*c* 0.1, water) and was crystallized from ethanol;

m.p. 94–95°, mixed m.p. 95–96°, $[\alpha]_D^{25}$ +83° (*c* 0.1, water); lit.[32]: m.p. 96°, $[\alpha]_D^{25}$ +84° (water).

D. 2,3,4-Tri-O-methyl-D-glucopyranose. — The fraction having R_{TMG} 0.55 (405 mg) showed by paper chromatography (solvents C and D), two components, corresponding to 2,3,4- and 2,4,6-tri-*O*-methyl-D-glucopyranose (small amounts). Due to overlapping, 2,4,6-tri-*O*-methyl-D-glucopyranose could not be determined quantitatively. 2,3,4-Tri-*O*-methyl-D-glucopyranose was characterized by formation of the *N*-(*p*-bromophenyl)-D-glucosylamine[34], m.p. 199–199.5°, $[\alpha]_D^{25}$ −145° (*c* 0.15, pyridine); lit.[34]: m.p. 198°, $[\alpha]_D^{25}$ −146° (pyridine).

E. 2,4-Di-O-methyl-D-glucopyranose. — The fraction having R_{TMG} 0.1 (24.3 mg) showed $[\alpha]_D^{25}$ +73° (*c* 0.1, water); lit.[33]: $[\alpha]_D$ +76.5° (water). Upon paper chromatography (solvents C and D), a single component having a mobility identical with that of 2,4-di-*O*-methyl-D-glucopyranose was detected. The component was characterized by formation of the *N*-(*p*-bromophenyl)-D-glucosylamine[33]; m.p. 242–242.5°, $[\alpha]_D^{25}$ −146° (*c* 0.4, pyridine); lit.[35]: m.p. 243–244°, $[\alpha]_D^{25}$ −147° (*c* 0.5, pyridine).

Partial acid hydrolysis of Tibi dextran. — Tibi dextran (1 g) was refluxed in 0.5M sulfuric acid (50 ml) for 2 h. The solution was cooled, neutralized with barium carbonate, and evaporated *in vacuo*. Chromatography in solvents A and B showed glucose and isomaltose. Isomaltose was separated by thick-paper chromatography (solvent A); yield: 0.103 g; $[\alpha]_D^{25}$ +99° (*c* 0.5, water); lit.[35]: $[\alpha]_D^{24}$ +103.2° (water). Isomaltose was characterized by formation of the β-octaacetate, m.p. 140–142°, $[\alpha]_D^{25}$ +94° (*c* 0.4, chloroform); lit.[35]: m.p. 143–144°, $[\alpha]_D^{25}$ +96.6° (*c* 2.7, chloroform).

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THE PECTIC SUBSTANCES OF *Zosteraceae*

PART II*. THE STRUCTURAL FEATURES OF THE PECTIC ACID

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ABSTRACT

The pectic acid obtained on partial hydrolysis of zosterine, which is a pectic polysaccharide of various *Zosteraceae* plants, has been shown to be a linear galacturonan containing (1→4)- and, possibly, (1→5)-linked residues of D-galacturonic acid.

INTRODUCTION

In a previous communication[1], we reported on a pectic polysaccharide, zosterine, isolated from various seaweeds of the *Zosteraceae* family found in the Sea of Japan. We now describe further data on zosterine and the structural features of the pectic acid prepared by its partial hydrolysis with acid.

EXPERIMENTAL

General experimental conditions. — Partition chromatography was performed on Whatman 3 MM or Leningrad factory "Goznak" paper with the following solvent systems (v/v): (*A*) ethyl acetate–pyridine–acetic acid–water (5:5:1:3); (*B*) butanone saturated with dilute ammonia; (*C*) butyl alcohol–acetic acid–water (4:1:5, upper layer). Thin-layer chromatography (t.l.c.) was performed on silica gel "KSK" (> 200 mesh) with chloroform–methanol (9:1) as developer. The detection reagents were as follows: (*1*) aniline hydrogen phthalate; (*2*) silver nitrate–phenol; (*3*) conc. sulphuric acid or saturated, aqueous ammonium sulphate (115°/10 min). Molecular weights were determined as described elsewhere[2]. All solutions were evaporated *in vacuo* at 30–40°. Uronic acid was determined by the decarboxylation procedure[3]. Ash content was estimated by heating polysaccharide samples to constant weight at 600°. Gas–liquid chromatography (g.l.c.) was performed as previously described[4].

Origin of specimens. — *Zosteraceae* plants were collected at the sublittoral of the Sea of Japan in the summer of 1967. Zosterine was obtained from fresh and air-dried plant material[1].

*For Part I, see Ref. 1.

Preparation of the pectic acid. — Zosterine (5.0 g) was hydrolysed with 10% sulphuric acid (1 litre) for 2 h at 95°. The precipitate formed was collected by filtration, washed with water until free of sulphuric acid, and dissolved in dilute ammonia. The solution was dialysed against water, and the pectic acid was reprecipitated with hydrochloric acid, deionised by dialysis, and freeze-dried to yield *ca.* 2.5 g of white powder, $[\alpha]_D^{20}$ +200° (dilute ammonia) (Found: mol. weight, 18,100; galacturonic acid, 98.3; methoxyl, 1.68; ash, 0.28; nitrogen, 0.0%). On complete hydrolysis with 2N sulphuric acid for 16 h at 95°, the pectic acid afforded D-galacturonic acid and only a trace amount of an unidentified sugar having an R_F value identical with that of apiose or rhamnose [paper chromatography in solvent (*A*), and t.l.c.[5]].

Periodate oxidation of the pectic acid. — The pectic acid (100 mg) was oxidized with periodic acid, followed by hydrolysis and oxidation with conc. nitric acid[6]. Paper chromatography of the reaction products [solvent (*C*)] revealed the presence of tartaric and oxalic acids in a molar ratio of *ca.* 1:1.

Preparation and analysis of the methylated galactan. — The pectic acid (1.3 g) was completely dissolved in 30 ml of methyl sulphoxide with vigorous stirring. Methylsulphinyl carbanion, prepared[7] from 0.7 g of sodium hydride and 20 ml of freshly distilled methyl sulphoxide, was added to the solution of pectic acid, and the mixture was kept for 6 h at room temperature in a nitrogen stream. Methyl iodide (15 ml) was added to the reaction mixture which was then stirred overnight. The partly methylated pectic acid was reduced with potassium borohydride (1 g) for 4 h at 10°. The mixture was then diluted with water and exhaustively extracted with chloroform, and the residual solution was dialysed until free of methyl sulphoxide and inorganic ions. The chloroform and aqueous solutions were combined and evaporated. The above procedure was repeated with the same amounts of reagents, followed by methylation as above but with omission of the reduction. The methylated and reduced galactan was recovered in the usual way[8], giving a chloroform solution (50 ml) which was poured slowly into light petroleum (300 ml). The precipitate was dried *in vacuo*, giving 0.5 g of a white material which had a methoxyl content of 39.5%. The i.r. spectrum showed the presence of only trace amounts of hydroxyl and carbonyl groups. The fully methylated galactan (0.2 g) was methanolysed with a mixture of 72% perchloric acid and methanol (1:10) for 3 h in a sealed tube at 100°, yielding the mixture of methyl glycosides, which was divided into two portions. The first portion was neutralized with Dowex-1 (HCO_3^-) resin, filtered, and evaporated. The residue was dissolved in chloroform and used directly for g.l.c. The second portion was diluted with water and heated in a sealed tube for 3 h at 100°. After neutralization with Dowex-1, the hydrolysate was examined by paper chromatography [solvent (*B*)] and t.l.c. For additional identification of methylated sugar derivatives, methylated galactan (0.3 g) was subjected to methanolysis as above to give a syrup (0.2 g) of methyl glycosides which was applied to the top of a column containing silica gel "KSK" (not exceeding 200 mesh). Gradient elution with increasing concentrations of ethanol in chloroform afforded a number of fractions containing pure methyl 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-galactoside and 1,2,3,5,6-penta-*O*-

methylgalactitol which had retention times identical with those of authentic samples. Hydrolysis of the methyl galactosides furnished the corresponding methyl ethers of D-galactose, which were identified[9] by comparison with authentic samples.

RESULTS AND DISCUSSION

The pectic acid examined in the present work was prepared by hydrolysis of short duration with 10% sulphuric acid. In this case, pure pectic acid (50%) was obtained as a white powder. The preparation was accompanied by partial splitting of the pectic acid to fragments of low molecular weight, and their removal was achieved by dialysis. The uronic acid content of zosterine (60%) corresponded with the yield of the pectic acid. The uronic acid content determined earlier[1] by the carbazole method was lower, but it is known[10-12] that large differences between carbazole and decarboxylation values are observed, particularly for polysaccharides of biological origin, and Dische[10] has stated that the carbazole reaction cannot be used for accurate determination of the hexuronic acid content of polysaccharides.

Almost complete cleavage of D-galacturonic acid residues was observed on periodate oxidation of the pectic acid, indicating the absence of (1→3)-linked residues and branch points. Hydrolysis of periodate-oxidised material and oxidation with concentrated nitric acid afforded oxalic and tartaric acids in the molar ratio of *ca.* 1:1. Isolation of the latter acid demonstrated the presence of (1→4)- or (1→5)-linked D-galacturonic acid residues in the pectic acid. The most substantial information about the pectic acid structure was afforded by methylation studies. Methylation of polysaccharides containing a high proportion of uronic acid residues may be difficult to achieve[13], and usually esterification and reduction to give a neutral polysaccharide is carried out before methylation[14]. However, these heterogeneous processes are not very effective and require multiple repetition and are thus time-consuming. In the present work, the very effective methylation procedure described by Hakomori[8] was used, followed directly by reduction with potassium borohydride and a second methylation, to give a methylated, reduced galactan containing only trace amounts of free hydroxyl groups (i.r. spectrum). It should be noted that the success of the methylation procedure was critically dependent on the maintenance of anhydrous conditions. The most satisfactory results were obtained with freeze-dried polysaccharides. The Hakomori methylation procedure[15] is accompanied by splitting of the chains of uronic acid residues due to the strongly basic conditions, and we have noted a similar effect. Thus, the methylated galactan could be fractionated into two fractions differing in molecular weight. The fractions from one polysaccharide were each subjected to methanolysis, followed by hydrolysis, and afforded qualitatively similar mixtures of methylated sugars.

Monosaccharide derivatives having the paper-chromatographic mobilities of the following compounds were formed from methylated galactan: 2,3,6-tri-*O*-methyl-D-galactose (main component) and 2,3,4,6-tetra-*O*-methyl-D-galactose (traces). The presence of 1,2,3,5,6-penta-*O*-methylgalactitol was revealed by t.l.c. Derivatives of

D-galactose having a lower extent of methylation were absent, thus demonstrating the absence of branching. Application of g.l.c. to the products of methanolysis of the methylated galactan revealed components having the retention times of methyl glycosides of 2,3,4,6- and 2,3,5,6-tetra- and 2,3,6-tri-*O*-methylgalactose and, possibly, 2,3-di-*O*-methylgalacturonic acid. A peak for 1,2,3,5,6-penta-*O*-methylgalactitol was also detected. It was noteworthy that methyl 2,3,6-tri-*O*-methylgalactoside was present in pyranose and furanose forms with a molar ratio of *ca.* 2:3. To confirm that a furanose form of galactose was present in the methylated galactan, and not produced during methanolysis, the mixture of methyl 2,3,6-tri-*O*-methyl-D-galactosides obtained from the methylated galactan was permethylated[16] to give a mixture of methyl 2,3,4,6- and 2,3,5,6-tetra-*O*-methyl-D-galactosides in the same molar ratio of *ca.* 2:3. On the other hand, treatment of an authentic sample of 2,3,6-tri-*O*-methyl-D-galactose with perchloric acid in methanol, as on methanolysis, followed by permethylation[16], gave methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside as the main product with only a small proportion of the corresponding furanoside. These data demonstrate the presence of furanose forms in the methylated galactan and, possibly, in the pectic acid, although there are indications that the furanose forms can be produced during methylation and the subsequent reduction of the pectic acid[17].

Therefore, all of the above results demonstrate the linear nature of the galactan and accordingly of the pectic acid, and point to the presence of (1→4)- and, possibly, (1→5)-linked sugar residues. The high specific rotation of the pectic acid indicated the α-D configuration of glycosidic bonds.

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THE SUBSTITUTION PATTERNS OF *O*-(2-HYDROXYETHYL)STARCH AND *O*-(2-HYDROXYETHYL)DEXTRAN

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ABSTRACT

The distributions of substituents in *O*-(2-hydroxyethyl) derivatives of starch and dextran have been examined by gas–liquid chromatography of the hydrolysates. Significant quantities of 1,2-*O*-ethylene-α-D-gluco-furanose and -pyranose (possibly together with the β-D forms) were found in the hydrolysates. The proportions of these products were dependent on the duration of the hydrolysis.

INTRODUCTION

O-(2-Hydroxyethyl) derivatives of polysaccharides have attained considerable industrial importance. The valuable properties of *O*-(2-hydroxyethyl)starch, in particular, have led to a wide range of applications[1]. Recently, considerable interest has been expressed in its use as a plasma volume expander[2].

The distribution of *O*-(2-hydroxyethyl) substituents in polysaccharides has been investigated by various methods. Thus, *O*-(2-hydroxyethyl)amylose of D.S. from 0.3 to 1.1 has been examined[3] by periodate-oxidation and tritylation techniques, the Smith degradation has been applied[4] to an *O*-(2-hydroxyethyl) derivative of corn starch (mostly amylose), and hydrolysates from an *O*-(2-hydroxyethyl)amylose and an *O*-(2-hydroxyethyl)cellulose have been examined by g.l.c.[5] and carbon-column chromatography[6], respectively.

Taking into account the cyclisation of 2-(2-hydroxyethyl) ethers of D-glucose[7], we are now able to interpret the gas–liquid chromatograms of the hydrolysates with greater confidence. In this paper, the analyses of *O*-(2-hydroxyethyl) derivatives of a waxy-maize starch (>99% of amylopectin) and of dextran (*Leuconostoc mesenteroides* B 512, $\overline{M}_w$ 70,000) are reported.

DISCUSSION AND RESULTS

Examination of the gas–liquid chromatograms of hydrolysates from *O*-(2-hydroxyethyl) derivatives of dextran or starch revealed the presence of various quantities of 1,2-*O*-ethylene-α-D-glucofuranose[7] and 1,2-*O*-ethylene-α-D-glucopyranose[7,8], together with the β-D isomers (see Fig. 1). Separation of the α- and β-D forms could not be effected on the columns investigated. It was apparent that the total and relative amounts of these bicyclic forms depended on the duration of the hydrolysis. Table I

contains the analyses of an *O*-(2-hydroxyethyl)starch after different periods of hydrolysis. It is evident that the proportion of cyclic forms increases with increasing time, with a consequent decrease in the proportion of 2-*O*-(2-hydroxyethyl)-D-glucose. The total amounts of 2-*O*-(2-hydroxyethyl)-D-glucose, and 1,2-*O*-ethylene-D-glucopyranoses and -glucofuranoses in the mixture remain approximately constant.

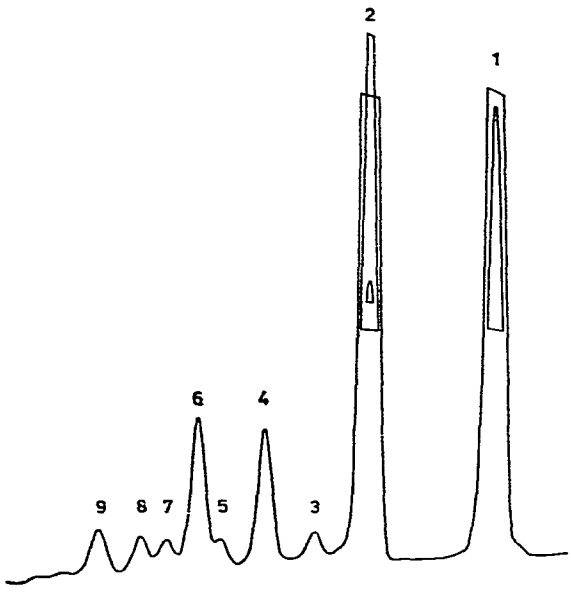


Fig. 1. Gas–liquid chromatogram of a hydrolysate (3 h) of *O*-(2-hydroxyethyl)starch: 1, α-D-glucose; 2, β-D-glucose; 3, 3-*O*-(2-hydroxyethyl)-D-glucose; 4, 2-*O*-(2-hydroxyethyl)-D-glucose; 5, 3-*O*-(2-hydroxyethyl)-D-glucose; 6, 2-*O*- and 6-*O*-(2-hydroxyethyl)-D-glucose; 7, 1,2-*O*-ethylene-α(or β?)-D-glucofuranose; 8, 6-*O*-(2-hydroxyethyl)-D-glucose; 9, 1,2-*O*-ethylene-α-D-glucopyranose (+β-D isomer?).

In the light of these results, the distribution patterns of *O*-(2-hydroxyethyl) derivatives of starch and dextran were examined. At the low D. S. chosen (0.2), the amounts of di-*O*-(2-hydroxyethyl) and poly(ethylene oxide) derivatives were negligible. Thus, it is possible to derive the following relative, initial rate-constants for the reaction of ethylene oxide with starch (mostly amylopectin) and dextran under the given conditions (see Experimental).

	k_2	k_3	k_4	k_6
Starch	6.6	1	—	1.2
Dextran	5.8	1	3	—

The low reactivity at O-6 in the hydrolysed amylopectin is consistent with the results of Srivastava *et al.*[4] who found that substitution occurred predominantly at O-2 in an *O*-(2-hydroxyethyl) derivative of corn starch (70% amylose). Other data[5] also indicate that the reactivity at O-2 is considerably greater than that at O-6.

TABLE I

O-(*2-Hydroxyethyl*) *derivative*	*Time of hydrolysis (h)*	*Mol. % of product*						
		D-*Glucose*	*2-HEG*[a]	*3-HEG*	*4-HEG*	*6-HEG*	*1,2-O-ethylene-*D*-glucofuranose*[b]	*1,2-O-ethylene-*D*-glucopyranose*[b]
Starch	3	85.2	8.2	1.7	—	2.1	1.1	1.7
Starch	5	85.3	7.0	1.8	—	2.1	1.0	2.7
Starch	7	84.0	6.3	2.0	—	2.6	1.9	4.3
Dextran	4	84.0	6.9	1.5	4.1	0.3	1.0	2.1

[a]2-HEG represents 2-*O*-(2-hydroxyethyl)-D-glucose, etc. [b]May consist of both α- and β-D-forms.

The relative reactivities of the hydroxyl groups in dextran towards ethylene oxide in 0.5% aqueous sodium hydroxide are also comparable with those obtained[9] during partial methylation in rather stronger alkali (19% NaOH) with methyl sulfate (k_2:k_3:k_4 = 8:1:3.5). Similarly, it has been shown that partial methylation of methyl α-D-glucopyranoside gives[10] comparable rate constants (k_2:k_3:k_4:k_6 = 7.8:1:4.3:13). We may infer from the overall similarity in reactivity patterns that the intrinsic reactivities of the hydroxyl groups in D-glucose towards these alkylating agents are retained in the polymer dextran[11].

At present, we can offer no explanation for the unexpectedly low reactivity of the primary hydroxyl group in starches. There are some indications that the reactivity at O-6 may be more dependent on alkali concentration than for the secondary hydroxyl groups[12].

EXPERIMENTAL

O-(2-Hydroxyethyl)dextran was prepared by treating dextran (*Leuconostoc mesenteroides* B 512, $\overline{M}_w$ 70,000) in aqueous 0.25% sodium hydroxide with ethylene oxide at 40°. Likewise, a hydrolysed waxy-maize starch (*ca.* 99% of amylopectin) was converted into *O*-(2-hydroxyethyl)starch.

Hydrolyses. — The hydrolyses were performed at polysaccharide concentrations of 1–2% in 2N sulphuric acid on a boiling-water bath.

Gas–liquid chromatography. — G.l.c. was performed on an F & M 5750 instrument with an 8-foot steel column packed with 5% butanediol succinate polyester (BDS) on Chromosorb W (90–210°, 2°/min).

ACKNOWLEDGMENTS

We are indebted to Professor B. Lindberg and Dr. J. E. Höök for supplying reference samples of the *O*-(2-hydroxyethyl)-D-glucoses and the bicyclic D-glucosides. The skilled technical assistance of Mr. A. Persson and Mr. R. Andren is also acknowledged. We are grateful to the Directors of Pharmacia for placing the facilities of the company at our disposal during these investigations.

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NUCLEOPHILIC REPLACEMENT REACTIONS OF SULPHONATES

PART VI[1]. A SUMMARY OF STERIC AND POLAR FACTORS

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ABSTRACT

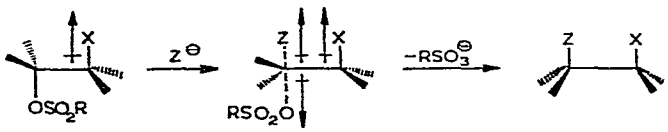
Since replacement reactions of sulphonic esters of carbohydrate derivatives normally proceed by an S_N2 mechanism, the formation of the highly polar transition state is subject to polar factors emanating from other electronegative substituents in the molecule. Consideration of these factors offers an explanation for the diminished reactivity of aldopyranoside 2-sulphonates, 1-sulphonates of hexulopyranose derivatives, and 6-sulphonates of galactopyranose derivatives. Furthermore, the presence of an axial, electronegative substituent adjacent to a sulphonate group on a pyranose ring inhibits replacement with charged nucleophiles, because of polar factors in the transition state of the reaction. Steric inhibition of displacement by a *β-trans*-axial substituent is also discussed.

INTRODUCTION

Sulphonic esters of carbohydrates have proved to be versatile intermediates because they undergo replacement reactions with a variety of nucleophiles[2], leading to the synthesis of amino sugars[3], thio sugars[4], deoxy sugars[5], etc. However, displacement of secondary sulphonate groups, particularly those attached to pyranose rings, with charged nucleophiles is not usually possible unless a dipolar, aprotic solvent, such as *N*,*N*-dimethylformamide, *N*-methyl-2-pyrrolidone, hexamethylphosphoric triamide, etc., is employed. The enhancement of reactivity in these solvents was originally attributed to lack of solvation of the nucleophilic anion by the highly basic solvent, thereby facilitating its nucleophilicity[6]. However, it has been shown recently that this solvent effect is more likely to be due to greater solvation of the transition state by the solvent, which lowers the activation enthalpy of the reaction[7]. Even in these solvents, the reactivity of a sulphonate group is critically dependent upon its position in the molecule, and, in the course of work on displacement reactions of sulphonic esters, we have found that it is often possible to predict the reactivity of a sulphonic ester by a consideration of the steric and polar factors affecting the formation of the transition state[1,8]. A summary of these conclusions is now presented. It must be emphasised that, because of the limited kinetic data available on these displacement reactions, this approach can only be regarded as qualitative.

DISCUSSION

The direct replacement of sulphonyloxy groups attached to carbohydrate moieties normally takes place by an S_N2 mechanism, as shown by the inversion of configuration which accompanies such reactions at asymmetric carbon atoms[2]. The geometry of the S_N2 transition state is well known, and its formation involves the generation of two highly polar bonds, one in the process of formation and the other in the process of degeneration. The formation of these polar bonds will be greatly affected by the presence of neighbouring polar substituents, and, if these are electronegative in character (*e.g.*, hydroxyl, halogen, etc.), the permanent dipole associated with the substituent-to-carbon bond should hinder the development of the transition state when an anionic nucleophile is used:

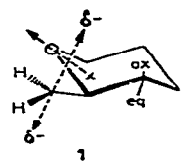


Such a hindrance will be at a maximum when the dipoles are antiparallel. On the other hand, electropositive substituents (*e.g.*, $\overset{\oplus}{N}R_3$) would show a reverse effect and should therefore enhance the reaction. Similarly, the use of a "neutral" nucleophile, such as ammonia or hydrazine, would reverse the polarity of the forming bond in the transition state, giving rise to favourable polar interactions with neighbouring, electronegative substituents. If we consider those sulphonic esters which are known to be somewhat resistant to replacement by charged nucleophiles, their diminished reactivity can be explained by consideration of the polar and steric factors in the transition state of the reaction.

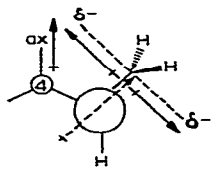
Hexopyranose 6-sulphonates. It is often assumed that nucleophilic displacement at primary positions is readily accomplished, regardless of other factors. However, the 6-sulphonates of galactopyranose derivatives are particularly resistant to replacement reactions, in comparison with the corresponding glucopyranosides [9-11]. This has been attributed to a polar field effect operating from the lone pairs of electrons on the axial O-4 and the ring-oxygen atom[9], but may be explained more precisely by a consideration of the polar, repulsive forces in the transition state.

The permanent dipole associated with the ring-oxygen atom bisects the C-1–ring-O–C-5 angle and lies in the plane of these three atoms with its negative end outside the ring. Formation of an S_N2 transition state at an exocyclic β-carbon atom would give rise to a polar, repulsive force with the ring-oxygen dipole, which would be minimised if the dipoles were held at 90°. Hence, the most favourable transition state would be **1**, which can best be visualised by viewing along the O-5–C-5 bond (**2**). From the projection formula **2** and molecular models, it can be seen that there should be no hindrance to the formation of this transition state, except when a large, electronegative, axial substituent is present at C-4. In such a case, there would be considerable steric overcrowding in the transition state and perhaps even an unfavourable dipolar

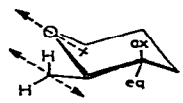
interaction between the C-4–O-4 dipole and the uppermost polar bond of the transition state, which are almost antiparallel. This may be relieved by rotation



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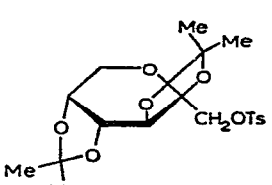
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of the C-6–C-5 bond, but, in this case, dipolar repulsion with the dipole of the ring-oxygen atom would come into play, leading to an unfavourable transition state **3**. The use of neutral nucleophiles greatly facilitates the displacement of galactose 6-sulphonates[9], presumably due to the change in polarity of the forming bond.

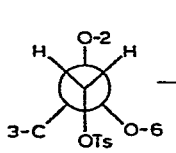
The resistance of other sulphonyloxy groups which are in similar environments to that in the galactopyranose 6-sulphonates has been noted, and the effect is probably quite general[11]. For example, the 1-benzenesulphonates of 2,4:3,5-di-*O*-benzylidene-L-xylitol and 2,4:3,5-di-*O*-methylene-DL-xylitol are both resistant to displacement by iodide[11], and 6-azido-1,3:2,4-di-*O*-benzylidene-6-deoxy-5-*O*-toluene-*p*-sulphonyl-D-glucitol does not readily undergo displacement with azide in *N*,*N*-dimethylformamide[12] under conditions in which most secondary sulphonyloxy groups attached to an acyclic portion of a molecule are readily replaced.

Hexulopyranose 1-sulphonates. The resistance of derivatives of hexulopyranose 1-sulphonates has been noted[13]. This may be explained[14] by consideration of the interactions between the permanent dipoles about the anomeric group with those formed during the development of the transition state. As an example, consider 2,3:4,5-di-*O*-isopropylidene-1-*O*-toluene-*p*-sulphonyl-β-D-*arabino*-hexulopyranose (**4**). The permanent dipoles of the anomeric group are best visualised by projection along the C-1–C-2 bond (**5**), and it can be seen that their presence gives rise to considerable dipolar repulsions in the two extreme transition states (**6** and **7**); hence, the formation of either would be unfavourable.

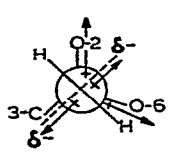
Similar explanations probably apply to the corresponding hexulofuranose 1-sulphonates, which are equally resistant to replacement, such that hexulofuranose 1,6-disulphonates undergo[13] ready, selective displacement at C-6, although, by analogy with the galactopyranose 6-sulphonates, one might expect a lesser reactivity from those in which the 4-substituent is *cis* to the sulphonyloxymethyl group.



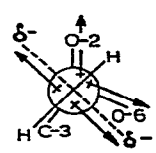
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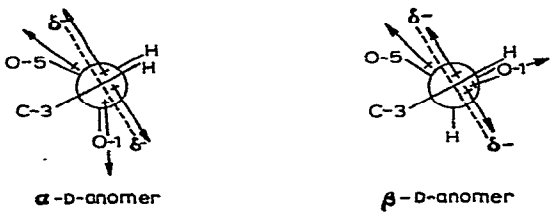


or



6 7

*Pyranoside 2-sulphonates**. Attempted replacement of a 2-sulphonyloxy group of a pyranoside with a charged nucleophile is normally unsuccessful, and, again, this may be ascribed to an unfavourable alignment of dipoles in the transition state of the reaction. The situation can best be visualised by viewing projections along the C-2–C-1 bond:



It can be seen[8,14] that, for the α-D anomer, both of the polar bonds of the transition state are inclined at an angle of *ca.* 30°, and in opposition, to the permanent dipoles of the C-1–O-1 and C-1–O-5 bonds, and clearly there should be considerable opposition to the formation of such a transition state. In the β-D anomer, the C-1–O-1 bond is perpendicular to the polar bonds of the transition state, and therefore the dipolar interaction should be at a minimum**. However, the uppermost polar bond of the transition state is inclined at 30° to the C-1–O-5 bond, and, consequently, considerable dipolar repulsion would still be expected, thus leading to a transition state of high energy. It appears, from this qualitative picture, that there should be less hindrance in the β-D anomer, so that, if displacement is to be achieved at the 2-position, the use of the β-D anomer might prove more successful.

The use of hydrazine, a neutral nucleophile, does result in displacement at the 2-position[15], and this must be due to the reversal of polarity of one of the polar bonds of the transition state, which would lead to a dipolar attractive force.

The only replacement reported at C-2 of a pyranoside with a charged nucleophile[16] is that of methyl 4,6-*O*-benzylidene-3-deoxy-2-*O*-toluene-*p*-sulphonyl-α-D-*ribo*-hexopyranoside which affords the 2-azide on treatment with sodium azide in *N*,*N*-dimethylformamide, but the conditions have not yet been specified. This reaction is obviously more favourable because of the absence of an electronegative substituent at C-3. The presence of a methylene group adjacent to a sulphonyloxy function has previously been shown to result in an enhancement of reactivity[17].

Pyranoside 3- and 4-sulphonates. The development of an S_N2 transition state at C-3 and C-4 is probably not greatly affected by the dipoles associated with the anomeric group but is mainly affected by steric and polar factors emanating from

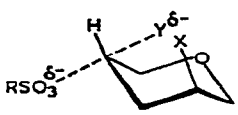
*In considering displacement reactions at carbon atoms which form part of a six-membered pyranoid ring, it is assumed that only slight deformation of chair and boat forms of that ring occurs during the formation of the S_N2 transition state.

**This should not imply that there is no repulsion between them, only that any such repulsion is at a minimum.

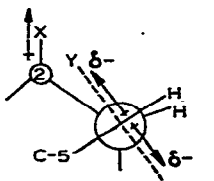
other groups in the ring. There are two factors which seem to affect displacement at these two positions:

(*a*) *The* β-trans-*axial substituent effect.* — When a *β-trans*-axial substituent is present with respect to a sulphonyloxy group, its displacement is impaired. For example, the α-anomer of 1,2,4,6-tetra-*O*-benzoyl-3-*O*-toluene-*p*-sulphonyl-D-glucopyranose does not undergo displacement with the benzoate anion in *N,N*-dimethylformamide, whereas the β-anomer does[18]. Similarly, methyl 2,3,6-tri-*O*-benzoyl-4-*O*-methanesulphonyl-α-D-mannopyranoside[19] (axial 2-benzoate) is resistant to replacement, whereas the corresponding glucopyranoside reacts[20]. This effect has also been observed with chlorosulphates[21]. Angyal *et al.*[22] have found, however, that methyl 2,4,6-tri-*O*-acetyl-3-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside does undergo a replacement reaction with benzoate under conditions (sodium benzoate in boiling *N,N*-dimethylformamide for 66 h) that would cause the majority of sulphonic esters to undergo extensive decomposition. Furthermore, we have observed that methyl 3-acetamido-2-azido-2,3-dideoxy-4,6-di-*O*-methanesulphonyl-α-D-altropyranoside undergoes a particularly ready replacement with azide anion, despite the axial group at C-2 in the *C1*(D) conformation. We have attributed this to the ease with which the altropyranoside should be able to change from the *C1*(D) to the *1C*(D) conformation in which there should be no hindrance to replacement[8].

The *β-trans*-axial effect is probably due to steric (and perhaps polar) factors in the transition state, and can be visualised with a pyranoside equatorial 4-sulphonate which has an axial group at the 2-position. The steric and polar clash in the transition state (**8**) is best seen by viewing along the C-4–C-3 bond (**9**). There is considerable steric interaction between the incoming nucleophile and the 2-substituent, giving rise to a transition state of high energy. It is also possible that there is some dipolar repulsion between the upper, polar bond of the transition state and the permanent



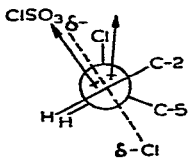
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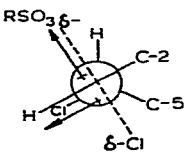
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dipole associated with the C-2–X bond, which are almost parallel with their negative ends converging. This is supported by the fact that methyl 6-deoxy-2,3-*O*-isopropylidene-4-*O*-toluene-*p*-sulphonyl-α-L-mannopyranoside undergoes direct replacement with hydrazine[23], but not with azide. When charged nucleophiles are used with this compound, ring contraction is observed as a result of participation of the *anti* C-5–O-5 bond[24], but it has not been established that such ring contractions are a general feature when displacements are attempted with sulphonates subject to the *β-trans*-axial substituent effect.

(*b*) *The effect of a vicinal axial substituent.* — The presence of a vicinal axial group which is *cis* to an equatorial chlorosulphonyloxy group on a pyranoid ring inhibits[21,25] replacement of that group. For example, whereas methyl 4,6-dichloro-4,6-dideoxy-β-D-glucopyranoside 2,3-di(chlorosulphate) readily undergoes displacement of the 3-ester group with chloride ion, the corresponding galactopyranoside (axial 4-chloro substituent) is resistant to replacement. This was interpreted as being due to an increase in steric interaction in the transition state of the reaction, since the departing sulphonate group moves closer to the axial group at C-4 and further away from the equatorial substituent[26]. However, we prefer[1,27] to regard this as mainly a polar effect, in which one of the dipoles of the transition state must become aligned with the permanent dipole associated with a neighbouring axial bond. This effect is best illustrated by viewing the transition states along the C-3–C-4 bond:

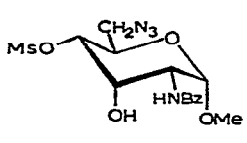


4-axial

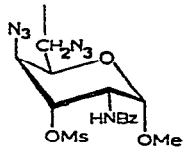


4-equatorial

It is clear that this effect should be observed whether the vicinal axial group is *cis* or *trans*, and this has been demonstrated. Thus, methyl 6-azido-2-benzamido-2,6-dideoxy-4-*O*-methanesulphonyl-α-D-allopyranoside (**10**), in which the 4-sulphonate group is adjacent to a *cis* axial hydroxyl group at C-3, is resistant to azide or benzoate ions in hexamethylphosphoric triamide, conditions under which the corresponding glucopyranosides (equatorial 3-group) readily react[1]. The failure to react readily under these conditions must be due to the axial substituent at C-3. A further example is provided by methyl 4,6-diazido-2-benzamido-2,4,6-trideoxy-3-*O*-methanesulphonyl-α-D-gulopyranoside (**11**), in which the axial sulphonate group at C-3 is adjacent to a *trans* axial azide group at C-4. Sulphonate **11** is likewise resistant to azide ion in hexamethylphosphoric triamide, due to the presence of the electronegative[28] azide group[1].



10

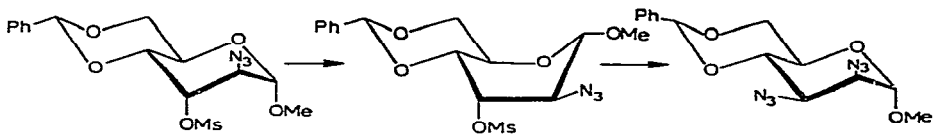


11

1,6-Anhydro-2,3-*O*-isopropylidene-4-*O*-methanesulphonyl-β-D-mannopyranose and -talopyranose are resistant[29] to azide ion under forcing conditions, and this lack of reactivity may be ascribed, at least in part, to the presence of an adjacent, axial, electronegative substituent at C-3.

One notable exception to this rule is the ease with which methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methanesulphonyl-α-D-altropyranoside (**12**) undergoes

replacement with sodium azide in *N,N*-dimethylformamide to give the 2,3-diazide **14**, despite the presence of the axial 2-azide group adjacent to the sulphonate residue[30]. However, there should be very little energy required for a change from the normal ground-state chair-conformation (**12**) to the boat conformation (**13**), in which there should be no hindrance to replacement.



12 13 14

By a consideration of the factors outlined in this paper, it should be possible to anticipate the reactivity of any sulphonic ester of a pyranoid derivative in an approximate and qualitative manner, but one must be careful to consider the possibility of the molecule reacting in an alternative conformation to that of the ground state. Furthermore, the size, as well as the electronegativity, of adjacent groups will probably affect the formation of the transition state as a result of a steric factor, but the importance of this, compared with the polar effect, is difficult to evaluate.

Whereas most substituents, excluding hydrogen atoms, attached to pyranoside rings are electronegative in character, the exact nature of the acylamido group is uncertain, because of the involvement of the lone pair of electrons on the nitrogen in resonance with the carbonyl group. This makes the nitrogen atom electropositive in character, thereby reversing the polarity of the ring-carbon-to-nitrogen bond, which would have a favourable effect upon the formation of an S_N2 transition state at a nearby carbon atom. Some preliminary indications are that the acetamido group does enhance the rate of replacement reactions at adjacent carbon atoms, and this aspect is receiving further attention.

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The author thanks Professors L. Hough and J. K. N. Jones, F.R.S., and Dr. D. H. Ball, for helpful discussions, and is indebted to Dr. Ball for access to the manuscript of a review on the reactions of sulphonic esters[31].

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ACYL MIGRATIONS IN ACYLATED MONOSACCHARIDES. FORMATION OF *N*-ACETYL-β-D-GALACTOFURANOSYLAMINE

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(Received January 6th, 1969)

ABSTRACT

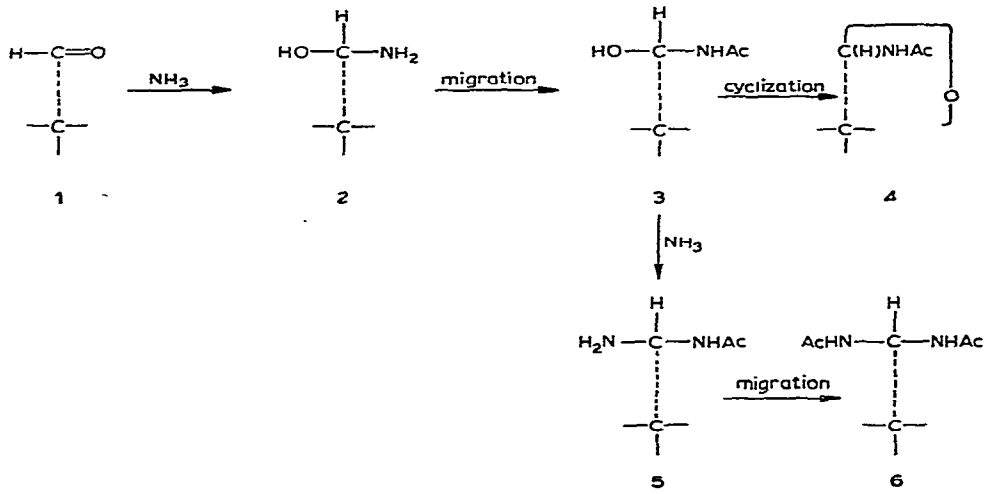
The conformational and steric factors that determine the different contributions of the acyl groups to the formation of 1,1-bis(acylamido)-1-deoxyalditols by the ammonolysis of acyl esters of carbohydrates of different structures are discussed. The isolation and structure of *N*-acetyl-β-D-galactofuranosylamine, obtained by such a reaction, are reported, and the conformation of this sugar and that of the *N*-acetyl-α-D-glucofuranosylamine previously described are considered on the basis of n.m.r. data.

INTRODUCTION

The reaction of acylated derivatives of mono- and di-saccharides with aqueous or alcoholic solutions of ammonia is very complex, and different products are formed by the following competitive reactions: (*a*) O→N acyl migrations, according to pathway **1**–**6**, to give the (acyclic) 1,1-bis(acylamido)-1-deoxyalditols (**6**) and the (cyclic) *N*-acylaldosylamines (**4**) having either furanoid or pyranoid structures[1,2]; (*b*) O→O acyl migrations on the chain of the sugar[3]; (*c*) ammonolysis of the acyl groups that esterify the parent sugar; (*d*) transesterification, which was evidenced when the reaction was performed in methanolic media[4]; and (*e*) formation of heterocyclic compounds[5].

The complexity emerging from the simultaneous operation of these different mechanisms makes particularized, qualitative consideration of each of them the best approach to interpretation of the experimental data recorded in the literature. Consequently, in this paper, the O→N acyl migrations that occur in this reaction will be discussed.

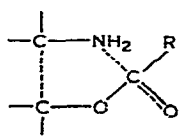
Starting from acylated *aldehydo* sugars, the formation of the bis(acylamido) compounds (**6**) and the *N*-acylaldosylamines (**4**) can be explained as resulting from an initial attack of ammonia on the carbonyl group of the sugar and successive migrations, as follows.



1 2 3 4 5 6

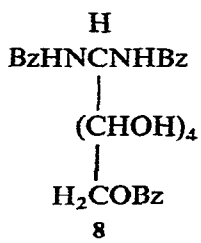
where Ac = acyl.

The tetrahedral intermediate **2**, possessing an amino group which has full availability of its nonbonded electrons, best explains the migration of an acyl group on the chain, to give **7**.



7

Many molecules of this intermediate can remain partially esterified as migration proceeds: this was shown experimentally by the isolation[6] of 1,1-bis(benzamido)-6-*O*-benzoyl-1-deoxyalditols (**8**), which demonstrated that the first acyl migration occurs when at least another two benzoyl groups are present.



8

DISCUSSION AND RESULTS

The contributions of differently situated acyl groups. — To ascertain the contributions of acyl groups situated at different positions on the chain to the formation of the bis(acylamido)alditols, studies were conducted with benzoates that possessed benzoyl groups labeled with carbon-14 on the carbonyl groups.

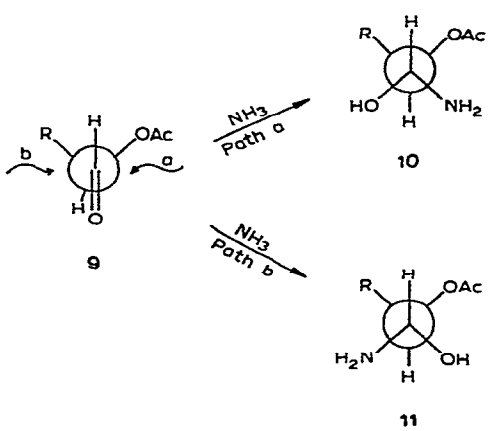
The radioactivity shown by the bis(benzamido) compounds (**6**) obtained by

ammonolysis of benzoates labeled at different positions of the chain, or of the ring, showed the apparent* contribution of each benzoyl group. These experiments were performed with D-glucose[1] and D-galactose[7] benzoates, and the following facts were established: (*a*) the benzoyl group on O-1 does not contribute to the formation of bis(acylamido) compounds, regardless of the starting benzoate; (*b*) in the acyclic, *aldehydo* benzoates (*e.g.*, 2,3,4,5,6-penta-*O*-benzoyl-*aldehydo*-D-glucose), the benzoyl group on O-2 contributes to a high degree (0.81 mole/mole)[8]; (*c*) for furanose and pyranose benzoates, the contribution of the 2-*O*-benzoyl group is small (approximately 0.12 mole/mole); (*d*) for pyranose benzoates, the contribution of the benzoyl group on O-3 is 0.80 mole/mole for D-glucose and 0.62 mole/mole for D-galactose; (*e*) the contribution of the benzoyl group on O-4 is 0.81 mole/mole for D-glucose and 1 mole/mole for D-galactose benzoates; (*f*) the contribution of the 6-*O*-benzoyl group is 0.30 mole/mole for D-glucopyranose benzoates and 0.18 mole/mole for D-galactopyranose benzoates.

The fact that the magnitude of the contributions of the benzoyl groups depends on the cyclic or acyclic structure of the starting products (which implies a different steric environment for the attack by ammonia) suggests that the reaction under discussion is *controlled by steric approach*[9]. The connection between this contribution and the direction of attack by ammonia can be seen by considering first the steric factors that influence this reaction for acyclic derivatives.

For this group of compounds, by rotation of the C-1–C-2 bond, there is obtained a set of conformations, of which, according to studies conducted with simple compounds[10], a conformation having the carbonyl group eclipsed with the hydrogen atom on C-2 (**9**) would appear to be the most probable.

Inspection of molecular models showed that the less hindered side for attack by ammonia is from the same side as the acyl group on O-2. If other conformations are considered for the starting aldehyde, a net preponderance of orientation **10** is obtained for the initial addition compound. After this stage, migration of an acyl group from the chain takes place.



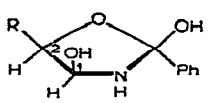
9 10 11

*It is considered "apparent", because a prior migration to another position cannot be excluded.

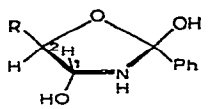
Lemieux[11] has pointed out that, if two hydroxyl groups can allow the formation of a cyclic acetal, it may also be expected that they will allow an acyl migration between them. This situation depends fundamentally on the existence of an adequate distance between the oxygen atoms of the two hydroxyl groups involved.

Although this idea was suggested for cyclic sugars, it is also reasonable for acyclic compounds. For alditols, Barker *et al.*[12] proposed a set of empirical rules for the formation of cyclic acetals, on the basis that the tetrahedral angles of the carbonyl carbon atom of the aldehyde or ketone employed are not appreciably changed in the acetal produced. From these rules, it follows that acetal formation is easier between O-1 and O-3 (β) than between O-1 and O-2 (α), and that, in turn, the latter is more probable than 1,4 (γ) acetal formation.

An acyl migration governed by the same factors that influence the formation of cyclic acetals* is supported by the similarity in bond lengths and bond angles[13], such that O and NH can replace each other in cyclic compounds without much change in the strain of the rings involved[14]. The migration of the acyl group on O-2 towards O-1 in the orientation **11** would lead to the intermediate **12**, having an unfavourable αC structure[12] which causes a strong *cis* effect (R and OH eclipsed in the transition state). Consequently, the molecules having the orientation **11** at C-1 would resist further change into migration products in which the benzoyl group on O-2 is involved, and would exhibit a tendency to follow other, competitive pathways.

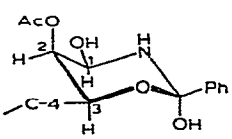


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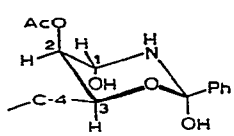


13

On the other hand, if the orientation **10** is considered, a better probability for the migration of the acyl group on C-2 is evident, owing to a more favorable, αT, cyclic structure (**13**). The high contribution of the acyl group on O-2 to the formation of the 1,1-bis(benzamido) compound from esters of *aldehydo* sugars indicates that a favored initial attack by ammonia occurs, giving the orientation **10** and, afterwards, the favored intermediate **13**. An example illustrative of the decisive importance of the steric factors discussed is found in the *N*-benzoylpseudoephedrine[15],



14



15

**In this case, these factors would be the distance between the atoms of the bridge O–C–N and the steric hindrances for the intermediate formation of the rings for the intramolecular migration of the acyl groups.

which affords a simple and definite case of acyl migration between O and N on neighboring carbon atoms.

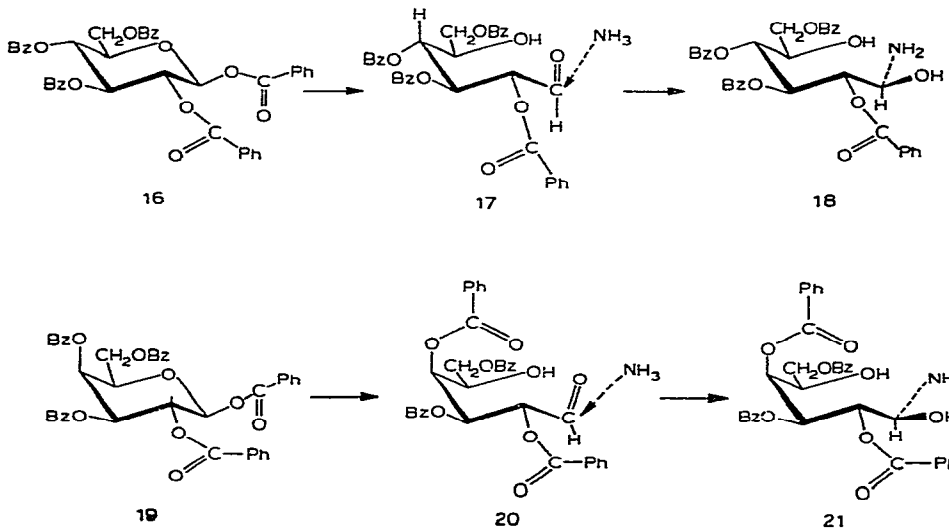
For the migration of the acyl groups on O-3, the differences in the steric hindrance in the cyclic intermediates **14** and **15**, which are formed from **10** and **11**, respectively, would be smaller than for a five-membered ring.

Once the first acyl group has migrated, these considerations again apply to the migration of the second acyl group, which would presumably be detached from O-2 or O-3, to give **6**.

In the case of furanose or pyranose esters, the scheme for the contributions of the various benzoyl groups changes as has been described. The data for the contributions in D-glucose benzoates (O-2, 0.11 mole/mole; O-3, 0.80 mole/mole, and O-4, 0.84 mole/mole) show a very low contribution from the acyl group on O-2, whereas that from the group on O-4 is exceedingly high for a poorly favored position (if an acyclic, planar zigzag structure is assumed). For D-galactose, the contributions present a similar pattern, but with an enhanced contribution from the benzoyl group on O-4 (1 mole/mole). The different contribution of the 2-*O*-benzoyl group of the cyclic esters (compared with that of the acyclic esters) can be explained on the assumption that, after a first step in which the ring opens and the carbonyl group is regenerated, the chain does not unfold inmediately and react, presumably as a puckered structure, with ammonia. Inspection of the conformational formulas and the molecular models of penta-*O*-benzoyl-*β*-D-glucopyranose (**16**) and penta-*O*-benzoyl-*β*-D-galactopyranose (**19**) revealed that the more accessible side for attack by ammonia on these cyclic molecules is the side opposite to the 2-*O*-acyl group. The first step would be the ammonolysis of the 1-*O*-benzoyl group, which would be eliminated as benzamide, and the opening of the hemiacetal ring would follow, with regeneration of the carbonyl group (see **16** and **17**). Assuming that **9** is the most favorable conformation for the C-1–C-2 rotamers, the favored direction of attack by ammonia is that opposed to the benzoyl group on O-2, if a puckered structure is maintained. In this structure, the peripheral distribution of the benzoyl groups is different from that present in the zigzag conformation of an acyclic structure; this situation affords a different steric environment for the attack by ammonia. The configuration at C-1 of the resulting D-glucose (**18**) and D-galactose (**21**) intermediates is analogous to that formulated in **11** as the more unfavorable for migration of the 2-*O*-acyl group.

The persistence of a puckered structure in the first stages of the reaction would also explain the high contribution of the acyl group on O-4, because, during the rotation of the bonds to form a zigzag, stretched structure, the molecule passes through a transient, helical conformation which brings the 4-*O*-acyl group and the amino group on C-1 close together. This situation is noteworthy in the D-galactose intermediate **20**, which has a 4-*O*-benzoyl group more adequately oriented with regard to the direction of attack by ammonia than that in the D-glucose intermediate **17**, thus accounting for the total contribution of 1 mole/mole of that benzoyl group in the ammonolysis of penta-*O*-benzoyl-*β*-D-galactopyranose.

The structures of the starting esters and the yields of bis(acylamido) alditols. — When 2,3,5,6-tetra-*O*-benzoyl-D-glucofuranose was ammonolyzed[1], a 62% yield of 1,1-bis(benzamido)-1-deoxy-D-glucitol was obtained. On the other hand, the ammonolysis of 3,5,6-tri-*O*-benzoyl-D-glucofuranose[16] gave only a 16% yield of the same compound. The difference between the two starting compounds of one benzoyl group, whose contribution is small, does not explain the great difference in yields. That this effect is related to the presence of a ring in the molecule is evidenced by the fact that 2,3,4,5,6-penta-*O*-benzoyl-*aldehydo*-D-glucose gave the bis(benzamido) sugar in a yield of 43%, and 3,4,5,6-tetra-*O*-benzoyl-*aldehydo*-D-glucose gave a 74% yield[16], showing that the free hydroxyl group on C-2 does not hinder migration, and that the presence of a benzoyloxy group on that carbon atom is not decisive as regards causing the reaction to take a favored migration pathway.



In general, high yields (50–78%) of 1,1-bis(acylamido)-1-deoxyalditols were observed in the ammonolysis of acylated *aldehydo* and furanose sugars, and lower yields (8–48%) from the corresponding pyranose esters. The different behavior shown by furanoid and pyranoid structures is attributable to the different rate of ring opening[17], which is low for the pyranose esters because of the greater stability; this allows mechanisms other than migration to compete during ammonolysis.

The reasons for the differences in rate of ring opening between furanose and pyranose structures has been discussed by Eliel *et al.*[18]. In general, the greater stability of the pyranose form is attributed to an easier accommodation of substituents. Since the steric interactions on the ring are relieved through the formation of an acyclic structure, a greater initial free-energy in the ground state (that would be present in the furanose compounds compared with the pyranose esters) would enhance the rate of ring opening.

In the ammonolysis reaction, 3,5,6-tri-*O*-benzoyl-D-glucofuranose would be more stable than 2,3,5,6-tetra-*O*-benzoyl-D-glucofuranose, owing to the absence from the former of an instability factor for the benzoyl group on O-2.

Formation of N-*acylaldosylamines.* — Some molecules having the intermediate structure **3** can cyclize to give *N*-acylaldosylamines of general structure **4**. Compounds of this type were isolated in low yield from sugar benzoates in the β-D-pyranose form[19,20]. On the other hand, in the ammonolysis of the acetates of D-glucose[21] and D-galactose, the formation of *N*-acylaldosylamines plays an important role. The ammonolysis of penta-*O*-acetyl-β-D-glucopyranose gave a high yield of *N*-acetyl-α-D-glucofuranosylamine (**22**) (41% in aqueous media)[22], whereas penta-*O*-acetyl-β-D-galactopyranose gave a 21% yield of *N*-acetyl-β-D-galactofuranosylamine (**23**) and 28% of the bisacetamido derivative. This difference in the yields of *N*-acetylaldosylamine (between the two sugar acetates) can be related to the different accessibility of the acyl groups on O-4 for migration, which, for the pentaacetate of D-galactose, favors the double migration to give the bisacetamido compound.

In the present paper, we report the isolation of *N*-acetyl-β-D-galactofuranosylamine and discuss its n.m.r. spectrum, as well as the spectrum of the *N*-acetyl-α-D-glucofuranosylamine already isolated[23].

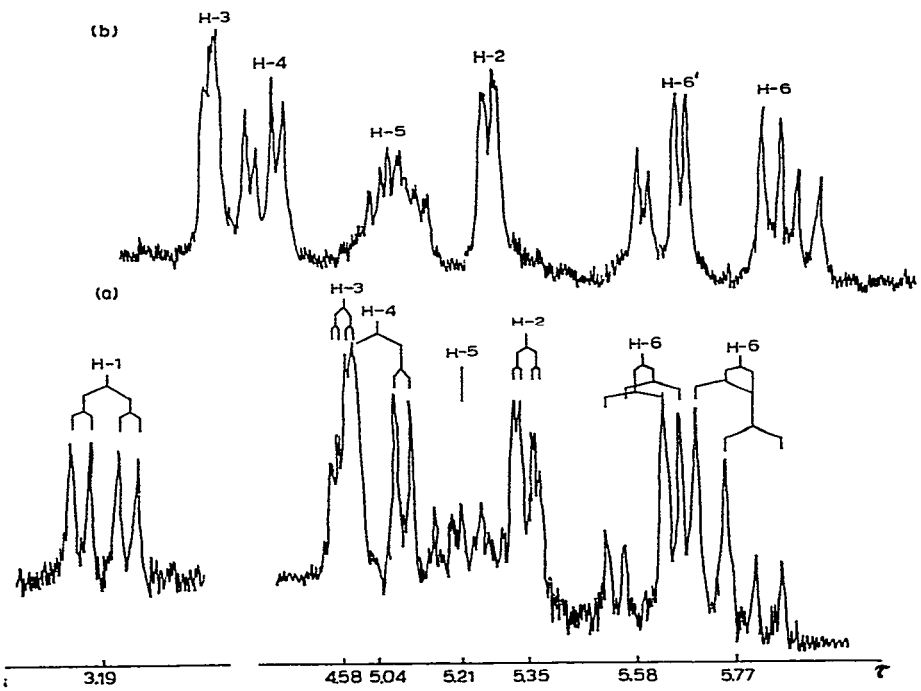


Fig. 1. The n.m.r. spectrum of *N*-acetyl-α-D-glucofuranosylamine (**22**) at (a) 100 MHz, and (b) 220 MHz, in pyridine-d_5.

The n.m.r. spectrum of *N*-acetyl-α-D-glucofuranosylamine (**22**) at 100 MHz in pyridine-d_5 (see Fig. 1a) is amenable to first-order analysis, although, for some

TABLE I

CHEMICAL-SHIFT DATA FOR COMPOUNDS **22** AND **23** IN PYRIDINE-d_5 AT 20° AND 100 MHz

Compound	*No.*	*Chemical shifts of protons* (τ)								
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*NH*	*N-Ac*
N-Acetyl-α-D-glucofuranosylamine	**22**	3.19	5.35	4.98	5.04	5.21	5.77	5.58	1.60	7.94
N-Acetyl-β-D-galactofuranosylamine	**23**	3.55	5.25	4.94	5.17	5.58	5.86		0.91	7.99

TABLE II

PROTON–PROTON COUPLING-CONSTANTS (Hz) FOR COMPOUNDS **22** AND **23** IN PYRIDINE-d_5 AT 20° AND 100 MHz

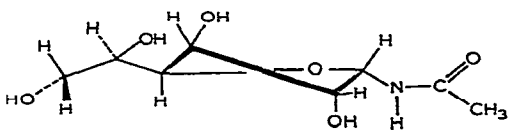
	No.	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	$J_{1,NH}$
N-Acetyl-α-D-glucofuranosylamine	**22**	4.0	1.0	3.0	8.0	5.5	3.5	11	10.5
N-Acetyl-β-D-galactofuranosylamine	**23**	4.5	4.5	5.7	2.6	6.0		—	9.0

points, a 220-MHz spectrum presents a clearer picture (see Fig. 1b). Chemical-shift data for compounds **22** and **23** are listed in Table I, and Table II gives the coupling constants. In the 220-MHz spectrum, each of the methine protons presents resonances well shifted from neighboring signals.

The anomeric proton (H-1) appears as a well defined pair of doublets due to coupling with H-2 and NH. When NH is irradiated, H-1 collapses to a doublet. The signal of H-2 is a partly resolved pair of doublets which, by irradiation of H-1, collapses to a doublet; simultaneously, NH also changes to a broad singlet.

In the 100-MHz spectrum, the H-3 signal is a pair of doublets partially superimposed on the H-4 pair of doublets, but both protons appear well differentiated in the 220-MHz spectrum. The signal for H-5 appears as a broad multiplet centered at τ 5.21; in the 220-MHz spectrum, it appears well shifted from adjacent signals. The methylene group gives rise to a pair of quadruplets separated by 11 Hz, and both protons give different coupling with the methine proton on C-5. The acetyl-group signal appears as an upfield singlet at τ 7.94.

By taking published studies[24–26] as a guide and considering the main non-bonded interactions present in **22**, some conclusions can be reached regarding its conformation. The values of $J_{1,2}$ and $J_{3,4}$ suggest a symmetric deformation of the furanose ring, which would be achieved by its assuming the T_2^3 conformation[27], shown in **22**.



22

Further considerations support this twist conformation for this compound. Application of a modified Karplus equation[25] gave the following dihedral angles: $\Phi_{1,2}$ 47°; $\Phi_{2,3}$ 68°; and $\Phi_{3,4}$ 54°. On comparing these values with those calculated by Abraham and McLauchlan[26] for the different modes of puckering of a cyclopentane ring, a reasonable agreement[28] between the experimental values and those tabulated for the twist conformation is observed. The lack of agreement between the value for the H-1–H-2 angle and the calculated values given[26] (which are lower) can be explained by the influence of the electronegative substituents on C-1, which diminish the coupling constant[29]. The value for $J_{1,2}$ thus requires some correction, owing to the deshielding effect of the oxygen atom and the *N*-acetylamino groups bonded to C-1; this correction would permit the placing of this coupling constant on the same basis as the remaining ones, which correspond to atoms bonded to only one oxygen atom. In some cases[30], it was considered reasonable to add 1.5 Hz to the experimental value for $J_{1,2}$, but, for the *N*-acetylaldosylamines, there is no comparative basis on which to make a sound modification. The value to be added to $J_{1,2}$ to bring the experimental value closer to the calculated one, would, in this case, be about 2–3 Hz.

The shape of the side chain would result from a statistical distribution, with a heavy proportional weighting of the forms of lowest energy. The favored shape can be predicted on the basis of the measured couplings if it is assumed that the *gauche* protons show couplings of 3–4 Hz, and the antiparallel protons show a spin–spin coupling[31] of 8–9 Hz. The high value of $J_{4,5}$ together with steric considerations suggest a *trans* relationship of H-4 to H-5, with a dihedral angle (average of divers rotamers) of about 160°. The value of $J_{5,6'}$ (3.5 Hz) suggest the predominance of a *gauche* relationship of H-5 to H-6′ in the conformational population, and $J_{5,6}$ (5.5 Hz) is large enough to indicate an almost antiparallel disposition of H-5 and H-6. The coupling constant for the methylene group gives a theoretical angle[32] of 111° between the geminal protons. Based on these data, the conformer depicted in **22** is a possible arrangement (which also presents the least steric interactions).

The high value for $J_{1,NH}$ suggests a *trans* relationship of both protons in a rotamer of restricted rotation, with little steric interaction between the *N*-acetyl group on C-1 and the other bulky substituents on the ring.

The structure of *N*-acetyl-β-D-galactofuranosylamine (**23**) was determined by periodate oxidation, which immediately gave 1 molar proportion of formaldehyde; the fast overoxidation that occurred is also characteristic of a furanose structure.

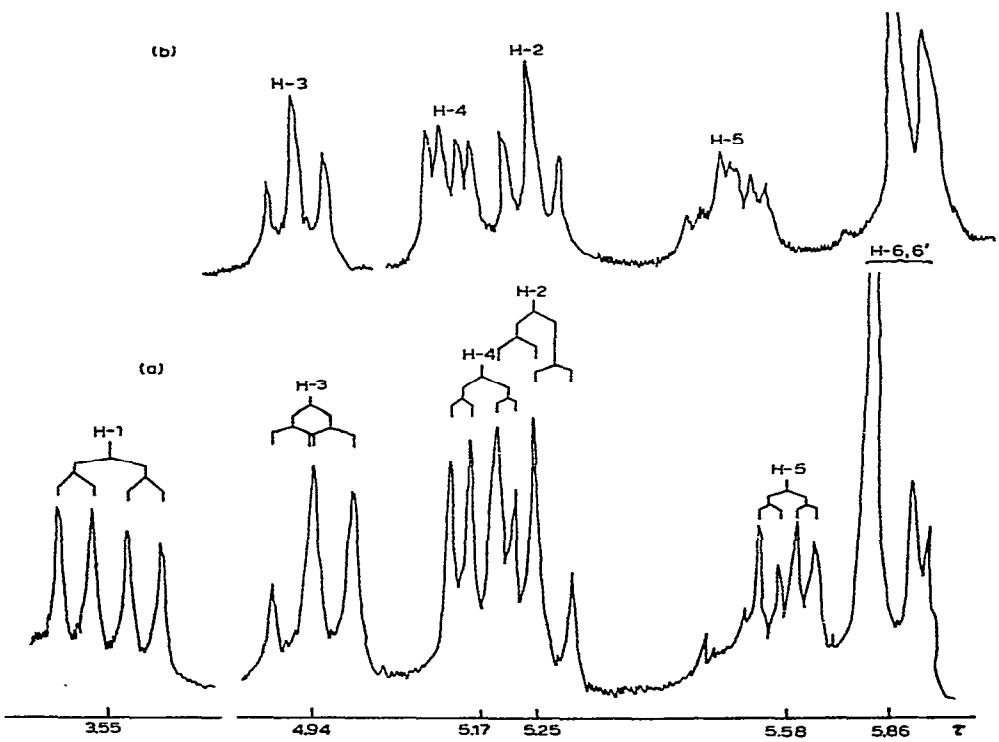


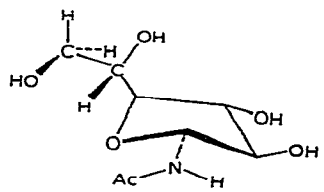
Fig. 2. The n.m.r. spectrum of *N*-acetyl-β-D-galactofuranosylamine (**23**) at (a) 100 MHz, and (b) 220 MHz, in pyridine-d_5.

The *beta* anomeric configuration assigned to this compound was based on optical rotatory data, through application of the Hudson rules of rotation[33].

In the 100-MHz spectrum of **23**, the H-1 signal appears as a well defined pair of doublets which collapse to a doublet on irradiation at the NH frequency. The signal for H-2 partially overlaps that for H-4, but irradiation of H-1 simplifies that multiplet, allowing the assignment of H-2. In the 220-MHz spectrum, both protons appear well separated. The chemical shifts and coupling constants are given in Tables I and II, respectively, and the spectra in Fig. 2.

Application of the modified Karplus equation[25] to the coupling constants of the ring protons, taking into account the configuration of the sugar, led to the dihedral angles $\Phi_{1,2}$ 132°; $\Phi_{2,3}$ 132°, and $\Phi_{3,4}$ 139°, indicating only a slight deformation from coplanarity. Inspection of molecular models revealed that an envelope conformation (V_2) best fits the calculated values. These values also agree with those tabulated for the buckling of a cyclopentane ring[26] in an envelope conformation.

The fact that, for compound **23**, a lowering effect of the electronegative NHAc group upon the coupling constant $J_{1,2}$ is not observed could be attributed to the full eclipsing of the σ (C-1–H) bond with a free *p*-electron orbital of the oxygen atom[34]. This effect usually increases the vicinal coupling constant (especially for J_{anti}) by about 2.3 Hz, which, in our case, would cancel out the electronegativity effect mentioned. For compound **22**, such eclipsing was not observed in molecular models.



23

The small $J_{4,5}$ coupling (2.6 Hz) shows that the preponderant conformer has a *gauche* arrangement of H-4 and H-5, as depicted in **23**. This conformation contains fewer nonbonded interactions with the remainder of the molecule. The value for $J_{5,6}$ and $J_{5,6'}$ suggests a major contribution from the conformer having H-5 and the methylene protons staggered.

EXPERIMENTAL

Paper chromatography was conducted on Whatman No. 1 paper, employing the following eluants, which also were used in cellulose-column chromatography: (A) 2:1:1 (v/v) butyl alcohol–ethanol–water; (B) 10:4:3 (v/v) butyl alcohol–pyridine–water; (C) 5:3:3:1 (v/v; top layer) ethyl acetate–pyridine–water–benzene. The evaporations were performed under diminished pressure below 60°. Melting points are not corrected.

Ammonolysis of penta-O-acetyl-β-D-galactopyranose. — (A) Isolation of 1,1-bis-

(*acetamido*)-*1-deoxy*-D-*galactitol.* Penta-*O*-acetyl-β-D-galactopyranose (20 g) was suspended in 400 ml of 25% aqueous ammonia, and dissolved by shaking for 1 h. After 24 h at room temperature, the solution was evaporated, and the residual solid was extracted with four 125-ml portions of ethyl acetate (to remove acetamide). The remaining solid was crystallized from ethanol, giving 2.2 g of 1,1-bis(acetamido)-1-deoxy-D-galactitol, m.p. 194–196°, which, after two recrystallizations from methanol, had m.p. 196–197°; $[\alpha]_D^{25}$ +6.5° (*c* 1.1, water); lit.[35] m.p. 195–197°, $[\alpha]_D^{25}$ +6.8° (water). Its peracetate had m.p. 200–201°; $[\alpha]_D^{25}$ −38.0° (*c* 1.01, chloroform); lit.[35] m.p. 200°, $[\alpha]_D^{20}$ −38.3° (chloroform). A further amount (1.8 g) was isolated by column chromatography as described under (*B*), giving a total yield of 28.2% of the bis(acetamido) alditol.

(*B*) *Isolation of* N-*acetyl*-β-D-*galactofuranosylamine* (**23**). The mother liquors from the 1,1-bis(acetamido)-1-deoxy-D-galactitol were evaporated, and the resulting syrup was chromatographed on a cellulose column (70 by 3.5 cm); elution was performed with solvent A, and 30 fractions (75 ml each) were collected. From fractions 13 and 14, a solid was obtained which, on recrystallization from butyl alcohol, gave 0.700 g of needles, m.p. 132–133°. After two recrystallizations from the same solvent, pure **23** was obtained, m.p. 133–134°; $]\alpha]_D^{20}$ −109.5° (*c* 1.07, water). A sample for analysis was dried at 100°/1 torr.

Anal. Calc. for $C_8H_{15}NO_6$: C, 43.44; H, 6.77; N, 6.33. Found: C, 43.75; H, 7.21; N, 6.62.

The remaining fractions (8.2 g) did not crystallize; they were combined, and chromatographed on a cellulose column (80 by 4 cm), by use of solvent B. Fifty fractions (50 ml each) were collected; from fractions 15–25, needles (990 mg) of m.p. 126–128° were isolated which, on recrystallization from methanol, had m.p. 132–133°. From fractions 32–37, a further 1.8 g of 1,1-bis(acetamido)-1-deoxy-D-galactitol, m.p. 193-195°, was obtained. The fractions that did not crystallize from this chromatography were rechromatographed on cellulose, with solvent C; 50 fractions (60 ml each) were collected. From fractions 18–24, compound **23** (720 mg), m.p. 132–133° was isolated. The total yield of **23** was 2.35 g (20.7%).

N-*Acetyl-tetra*-O-*acetyl*-β-D-*galactofuranosylamine.* — Compound **23** (160 mg) was acetylated at room temperature with 16 ml of 1:1 pyridine–acetic anhydride. The solution was kept for 24 h at room temperature, and then evaporated over sulfuric acid in a vacuum desiccator. The resulting syrup was crystallized from 4 ml of 3:1 butyl alcohol–light petroleum (b.p. 60–80°), to give 260 mg of rectangular plates, m.p. 109–110°; recrystallized twice from the same solvent, it had m.p. 111–112°, $[\alpha]_D^{21}$ −15.7° (*c* 1.17, chloroform). A sample for analysis was dried at 100°/1 torr.

Anal. Calc. for $C_{16}H_{23}NO_{10}$: C, 49.35; H, 5.91; N, 3.59. Found: C, 49.43; H, 5.75; N, 3.67.

Structure of **23.** — The structure of compound **23** was determined by periodate oxidation. The spectrophotometric technique was applied, the uptake of sodium metaperiodate being determined[36,37] with a Beckman D. U. spectrophotometer at 222.5 nm[36,37], and the formaldehyde[38] at 570 nm. Oxidations were performed in

duplicate with samples (7 mg) of **23** dissolved in 3 ml of 100 mM sodium metaperiodate; the reaction mixture was kept at 20° during the oxidation. Formic acid was titrated with 10 mM sodium hydroxide. The results are given in Table III. The immediate production of one mole of formaldehyde, and the subsequent easy over-oxidation, indicated a furanose structure.

TABLE III

PERIODATE OXIDATION OF COMPOUND **23**

Time (*h*)	*$NaIO_4$* (*moles*)	*Formic acid* (*moles*)	*Formaldehyde* (*mole*)
0.3	1.41	0.00	0.93
1	2.24	0.12	0.93
4	3.34	0.51	0.93
24	5.50	2.16	0.93

Spectra. — N.m.r. spectra were recorded at 100 MHz at 20–25° with a HA-100 and at 220 MHz with a HR-220 Varian Spectrometer. Tetramethylsilane (τ 10.00) was used as the internal standard. Coupling constants (see Table II) were read directly from the spectra. Hydroxyl protons were interchanged by adding D_2O. The data on chemical shifts are given in Table I.

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THE CONFIGURATIONS AT THE ANOMERIC CARBON OF THE REACTION PRODUCTS OF SOME DIGESTIVE CARBOHYDRASES

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ABSTRACT

Pancreatic α-amylase liberates both maltose and maltotriose in the α form; barley β-amylase liberates maltose in the β form (as determined by g.l.c.). Intestinal lactase liberates D-galactose in the β-pyranose form (as determined by g.l.c. and enzymic analysis). Intestinal sucrase liberates D-glucose in the α form (as determined by enzymic analysis, confirming previous g.l.c. data). The lack of inversion at the anomeric carbon by pancreatic amylase, intestinal lactase, and sucrase is compatible with both a "front attack" and a "double displacement" mechanism.

INTRODUCTION

The interest in the identification of the anomeric configuration of the sugars liberated by an enzyme is at least two-fold: (*a*) any hypothesis on the reaction mechanism for the enzyme must take into consideration this piece of information; (*b*) in a metabolic chain of reactions, the anomeric configuration of sugars produced by an enzyme can usually act as substrates for the next step, which can be an enzymically-catalysed mutarotation or another reaction in a metabolic pathway (*e.g.* refs. 1, 2, 3). If this were not so, the relatively slow nonenzymic mutarotation, which is poorly controlled, would be the rate-limiting step in the metabolic pathway.

Polarimetry is the oldest procedure applied in the identification of the anomeric configuration liberated by a carbohydrase. Differential oxidation by bromine has also been used[4,5]. Recently, gas–liquid chromatography of sugars[6,7] and enzymes specific for one anomeric form[8] have been suggested for the same purpose. Both methods are much more sensitive than polarimetry, and g.l.c. has the further advantage of permitting identification of individual anomers in complex mixtures of sugars, whereas enzymic methods, once the appropriate enzyme is available, are the least time-consuming.

The following report describes an investigation of the anomeric configuration of maltose and maltotriose produced by pancreatic amylase, of maltose produced by barley amylase; of D-galactose produced by intestinal lactase, and of D-glucose produced by intestinal sucrase. Gas-liquid, chromatographic or enzymatic procedures were used.

The carbohydrases from small intestine and from pancreas did not cause any inversion of the configuration at the anomeric carbon. Our results are new in the

case of lactase, whereas they extend and confirm those obtained previously on the other enzymes with other procedures; they indicate once more, therefore, the reliability of g.l.c.[6,7] for this sort of investigation.

MATERIAL AND METHODS

Enzymes. — Hog pancreatic α-amylase (EC 3.2.1.1., twice crystallized, prepared according to Caldwell *et al.*[9]), and barley β-amylase (EC 3.2.1.2, 2000° Lintner units/g) were obtained from Mann Res. Lab., New York. Rat intestinal lactase (EC 3.2.1.23) was prepared according to Kerry, Hore, and Semenza[10]; it had a specific activity of 4 units/mg (with lactose as substrate). Rabbit intestinal sucrase (sucrose glucohydrolase)–isomaltase was prepared according to a modification of Kolínska and Semenza's procedure[11]; it had a specific activity of 14 sucrase units/mg protein. Glucose oxidase (type I, β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) and β-galactose dehydrogenase (β-D-galactose:NAD oxidoreductase), prepared from *Pseudomonas fluorescens* according to Wallenfels and Kurz[8] were obtained from Boehringer, Mannheim, West Germany. The latter enzyme had a specific activity of approximately 5 units/mg. Peroxidase from horse radish (type I) was obtained from Sigma, St.-Louis, Mo.

Other chemicals — Amylose was obtained from K & K Fine Chemicals, Plainview, N.Y. Maltotriose was a generous gift from Dr. Auricchio, Naples, Italy. Hexamethyldisilazane (purum) and chlorotrimethylsilane (puriss.) were purchased from Fluka AG, Buchs, Switzerland. All other chemicals were reagent grade. The purity of both (+)-lactose monohydrate (puriss., Fluka) and α-D-galactopyranose (puriss., Fluka) was checked by g.l.c. No D-galactose or D-glucose was detected in the lactose sample, and only traces of β-D-galactopyranose were detected in α-D-galactopyranose.

Gas-liquid chromatography. — The following instruments were used for g.l.c.: "F-20" Perkin–Elmer with flame ionisation detector; Aerograph 1520 with double-column system and temperature programming; or Fractovap model D (Carlo Erba) with flame ionisation detector.

The general design of the experiment was essentially the same as that described in a previous paper[6]: the sugar was incubated with or without enzyme in the absence of buffer in order to keep the rate of mutarotation low (see *e.g.* ref 4.). Aliquots were frozen at various times in liquid air, lyophilized, per(trimethyl)silylated in dry pyridine according to Bentley *et al.*[12], and analyzed by g.l.c.

Incubation of amylases. — Each sample contained, in 0.5 ml of water, amylose, (0.25 mg), calcium chloride, (0.75 μmole) and amylase (either from pancreas or from barley, 2.5 mg). The samples were frozen after incubations of 1, 2, 4, and 20 min at 37°, and processed as reported above. For per(trimethylsilyl)ation of each sample, 0.2 ml of the reagent (17:2:1 pyridine–hexamethyldisilazane–chlorotrimethylsilane) was used. Chromatography conditions: Carbowax 20 M, 20% column; Tc: 223°; Tj: 265°; nitrogen, 2.4 atm, 55 ml/min. This procedure separated the two anomers of maltose (see Table I). The results are shown in Fig. 1.

For the identification of the anomeric configuration of maltotriose, a much smaller amount of pancreatic α-amylase was used. Each sample contained in 0.5 ml water: amylose, 0.25 mg; calcium chloride, 0.75 μmole; pancreatic α-amylase,

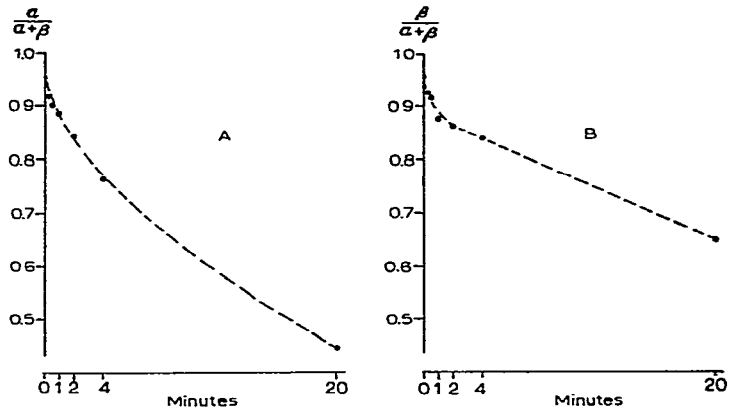


Fig. 1. Composition of the maltose released during the degradation of amylose by pancreatic α-amylase (A), and by barley β-amylase (B). α, Concentration of α-maltose; β, concentration of β-maltose.

TABLE I

RETENTION TIMES IN THE G.L.C. OF THE PER(TRIMETHYLSILYL)ATED DERIVATIVES OF THE VARIOUS ANOMERS OF D-GLUCOSE, D-GALACTOSE, MALTOSE, AND MALTOTRIOSE[a]

Compounds	*SE-30, 2%, on Chromosorb G*[b]	*Carbowax 4000, 3%, on Gaschrom P*[b]	*Carbowax 20M, 20%, on Gaschrom P*[b]	*SE-30, 1%, on Gaschrom P*
			min	min
α-D-Glucopyranose	1	1		
β-D-Glucopyranose	1.8	1.86		
α-D-Galactopyranose	0.89	0.86		
β-D-Galactopyranose	1.10	1.20		
β-D-Galactofuranose	0.75	0.71		
α-Maltose			17.6	
β-Maltose			21.0	
α-Maltotriose				17.0
β-Maltotriose				20.8

[a]For details, see under "Methods". [b]Relative to α-glucose.

0.25 mg. The samples were frozen after incubations of 0.5, 1, 2, 4, 10, and 20 min at 37°. Per(trimethylsilyl)ation was performed overnight with 0.2 ml of the reagent for each sample. As maltose and maltotriose were determined in different chromatograms, the ratio between the two is only approximate. The chromatographic separation of the two anomers of maltotriose was incomplete, but it was sufficient for a quantitative analysis (Fig. 2). Chromatography conditions: silicon column SE-30, 1% on Gaschrom P (80–100 mesh); diameter 2.7 mm, height 1 m; Tc: 235° (for malto-

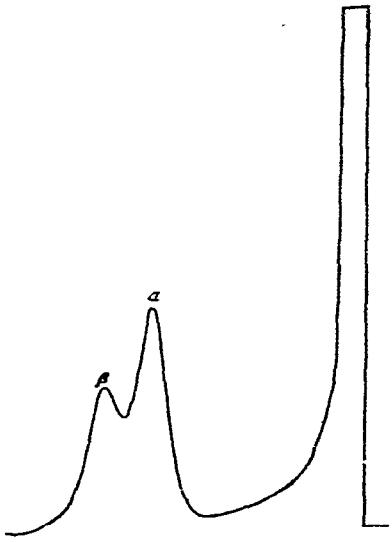


Fig. 2. Gas–liquid chromatographic separation of the per(trimethylsilyl)ated derivatives of α- and β-maltotriose. For details, see under "Materials and Methods".

triose) and 190° (for maltose); Tj: 300°; nitrogen, 84 ml/min. The results are shown in Fig. 3.

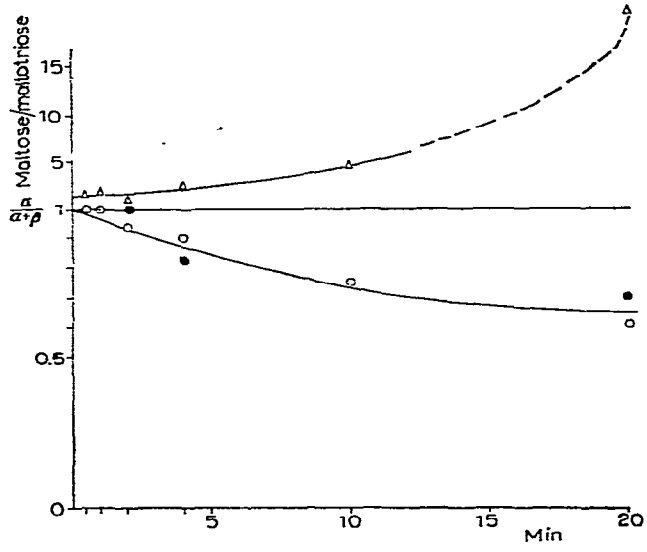


Fig. 3. Degradation of amylose by pancreatic α-amylase. Top figure: Ratio of total maltose to total maltotriose (△). Bottom figure: Composition of maltose (○), respectively maltotriose (●), coincidence of ○ and ● (⊙).

Incubation of lactase. — Calf intestinal lactase has a smaller Km for α-lactose than for β-lactose[13]. We have therefore used a lactose solution consisting preponder-

antly of the α-anomer. This explains the prevalence of α-D-glucose over β-D-glucose in the chromatograms (see Fig. 4).

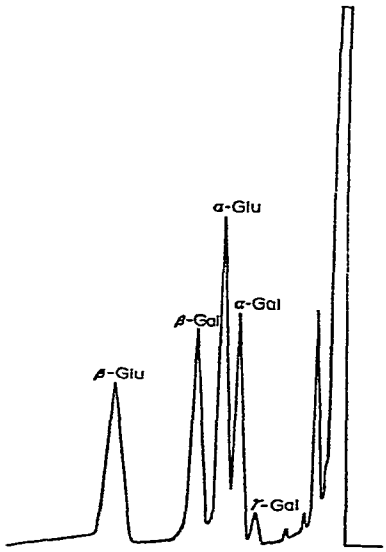


Fig. 4. Gas-chromatographic separation of the per(trimethylsilyl)ated derivatives of D-glucose and D-galactose formed during the hydrolysis of lactose by lactase: γ-Gal, β-D-galactofuranose; α-Gal, α-D-galactopyranose; β-Gal, β-D-galactopyranose; α-Glu, α-D-glucopyranose; and β-Glu, β-D-glucopyranose. The peaks appearing before γ-galactose are produced by pyridine and minor impurities present in the reagent. Column: Carbowax 4000, 3%. For details, see under "Materials and Methods".

α-Lactose (20 mg) was dissolved in water (1 ml) at 15.1°. Four lactase units were added. After 5, 10, 15, 20, 30, 45, 60, 180, and 300 min, samples (0.1 ml) were withdrawn and frozen. After lyophilisation, each sample was per(trimethylsilyl)ated with 0.2 ml of reagent (7:2:1 pyridine–hexamethyldisilazane–chlorotrimethylsilane). Chromatography conditions: silicon column SE-30, 2% on Chromosorb G (80–100 mesh), diameter 2 mm, height 80 cm; Tc: 154° and 150°; Tj: 200°; nitrogen: 0.6 atm, approx. 24–25 ml/min. Alternatively, the following chromatographic conditions were used: Carbowax 4000, 3%; Gaschrom P (80–100 mesh), height 2 m; Tc: 160°; Tj: 265°; nitrogen: 50 ml/min (see Fig. 4 and Table I).

Spontaneous mutarotation of α-D-galactose. — Crystalline α-D-galactopyranose (21.4 mg) was dissolved in water (2 ml) at 15.1°. After 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 min at this temperature, samples (0.1 ml) were withdrawn, frozen, and lyophilyzed. Per(trimethylsilyl)ation was performed with 0.1 ml of reagent (7:2:1 pyridine–hexamethyl disilazane–chlorotrimethylsilane), then pyridine (0.1 ml) was added, and the mixture kept overnight. G.l.c. was performed on SE-30, as described in the previous paragraph (Tc: 154°). The results as shown in Fig. 6.

Mutarotation of α-D-galactose in the presence of lactase. — Crystalline α-D-galactopyranose (10.2 mg) was dissolved at 15.1° in water (1 ml) containing 4 units

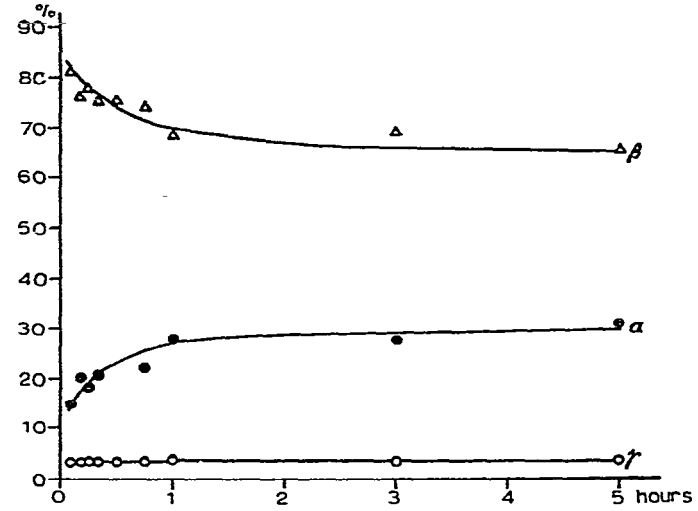


Fig. 5. Composition of D-galactose released from lactose by lactase at 15.1°; △, β-D-galactopyranose; ●, α-D-galactopyranose; ○, β-D-galactofuranose.

of lactase. Samples (0.1 ml) were withdrawn after 5, 10, 20, 30, 60, 180, and 300 min, and processed as above. The results are shown in Fig. 6.

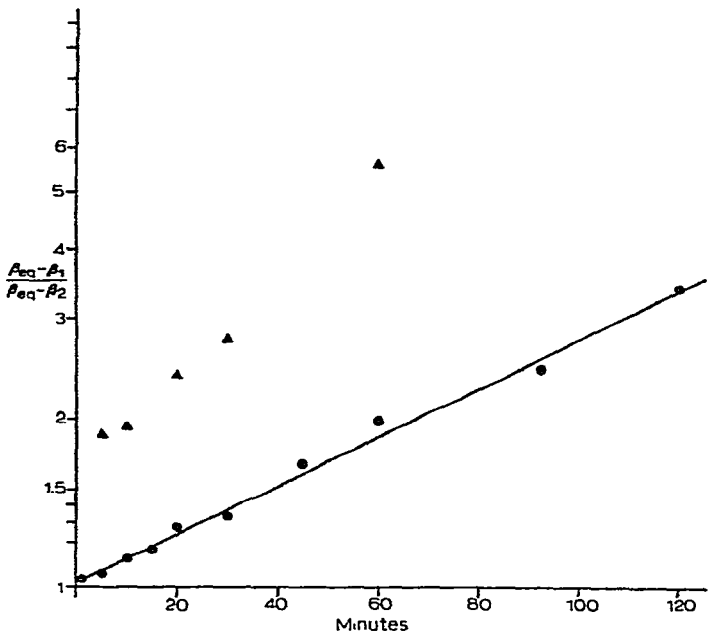


Fig. 6. Mutarotation of α-D-galactopyranose at 15.1° with (▲) or without (●) lactase, as determined by g.l.c.

Enzymic identification of the anomeric form of D-*galactose released by rat-intestinal lactase.* — All incubations were performed at room temperature (approx. 20°).

Lactase (0.7 unit) was incubated with lactose (86 mmoles) in 1.4 mM potassium phosphate buffer, pH 6.8, in a total volume of 0.35 ml. The incubation was stopped after 1 min by addition of M 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (Tris) buffer (0.1 ml), pH 8.6. A sample (0.1 ml) was immediately withdrawn and added to a semi-microcuvette containing, in a volume of 1 ml, NAD (0.5 mg), β-galactose dehydrogenase (1.0 mg, *ca.* 5 units), ammonium sulfate (0.4 mmole, present in the galactose dehydrogenase preparation), and 0.2 mmole of 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride buffer, pH 8.6. The change in absorbancy was recorded at 340 nm (Fig. 7, A).

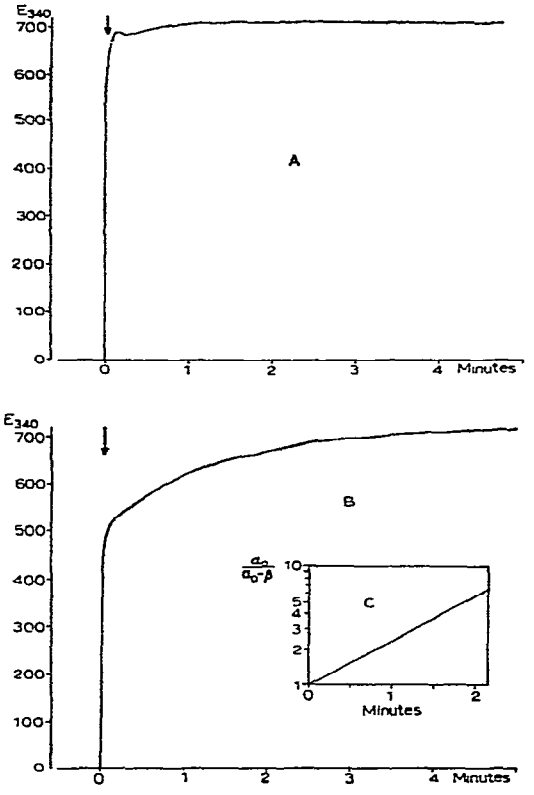


Fig. 7. Enzymic identification of the anomeric configuration of D-galactose liberated by lactase. The D-galactose liberated by lactase is dehydrogenated by β-galactose dehydrogenase rapidly and essentially to completion (A). After mutarotation of the lactose-lactase incubation mixture (B), only two thirds of the galactose present are rapidly dehydrogenated. The slow dehydrogenation of the last third follows a first-order kinetic (C). α_0, Concentration of the α-D-galactopyranose at the beginning of the slow phase; β, concentration of the β-D-galactopyranose formed from α-D-galactopyranose during the slow phase. For details, see text.

The remaining 0.35 ml of the lactose–lactase–buffer mixture were warmed for approximately 2 min at 90°, cooled to room temperature, and a sample (0.1 ml) examined exactly as before (B). It is apparent that in the nonmutarotated sample (A)

almost all the D-galactose is present as β, whereas in the sample having reached mutarotation equilibrium (B) about two thirds are quickly dehydrogenated; the rate-limiting reaction in the dehydrogenation of the last third is probably the mutarotation of other forms into β-D-galactopyranose. Assuming that this mutarotation is $\alpha \xrightarrow{k_1} \beta$, the velocity constant k_1 was calculated (C) as 0.88 min^{-1} (natural logarithms were used). α_0, initial concentration of α-D-galactose; β, concentration of β-D-galactopyranose which originated from α-D-galactopyranose.

Enzymatic identification of the anomeric form of D-*glucose released by rabbit-intestinal sucrase.* — The glucose oxidase–peroxidase reagent of Huggett and Nixon[14] and of Sols and De La Fuente[15] was modified in order to permit an easy identification of the anomeric configuration of D-glucose. Shortly before use, the following reagents were mixed in the following order: water (8.8 ml), 10 mM mercuric chloride (0.5 ml), M sodium acetate buffer (pH 5.0, 0.5 ml), glucose oxidase (Boehringer, type I) (5 mg), horse-radish peroxidase (5 mg), 20% Triton X-100 in ethanol (v/v) (0.2 ml), and 1% *o*-dianisidine in ethanol (w/v) (0.05 ml).

Mercuric chloride was added in order to inhibit the traces of mutarotase which may still be present in the enzyme preparations. At the concentration of 0.5mM, mercuric chloride completely inhibits mutarotase[5,2] as well as sucrase, but does not affect either glucose oxidase or peroxidase. Diluted sodium acetate buffer, pH 5.0, was used in order to keep the rate of mutarotation low[4].

Much larger amounts of enzymes were used than in the original methods[14,15] in order to increase the velocity of the enzymic reactions well above the velocity of mutarotation. It was ascertained that peroxidase was not the rate-limiting factor in the reaction sequence, and that essentially no sucrase activity was present in this reaction mixture.

All incubations were performed at room temperature (approx. 20°). Sucrase (17.5 units) was incubated with 50mM sucrose in a total volume of 50 μl. The incubation was stopped after 30 sec by addition of 0.13M 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride (50 μl), pH 5.2. A sample (30 μl) was immediately withdrawn and mixed in a semi-microcuvette with the glucose oxidase–peroxidase reagent (1 ml) described previously. The change in absorbancy at 430 nm was recorded. As a blank, the glucose oxidase–peroxidase reagent was used (Fig. 8, C). The remaining of the sucrase–sucrose–buffer mixture (70 μl) was warmed for approximately 2 min at 90°, cooled to room temperature, and a sample (50 μl) was mixed with fresh glucose oxidase–peroxidase reagent, as described previously (Fig. 8, D).

It is apparent that the D-glucose present in the nonmutarotated sample was oxidized very slowly by glucose oxidase (C), whereas the D-glucose present in the sample having reached mutarotation equilibrium was oxidized very quickly (D). Fig. 8 also shows the rate of oxidation of comparable amounts of α-, respectively β-D-glucopyranose (A, B).

RESULTS AND DISCUSSION

Pancreatic α-amylase. — In 1925, R. Kuhn[16] showed that the products of

the degradation of amylose by pancreatic amylase mutarotate downwards, and concluded that maltose (at the time believed to be the only reaction product) was released by pancreatic α-amylase in the α-configuration. His polarimetric data have

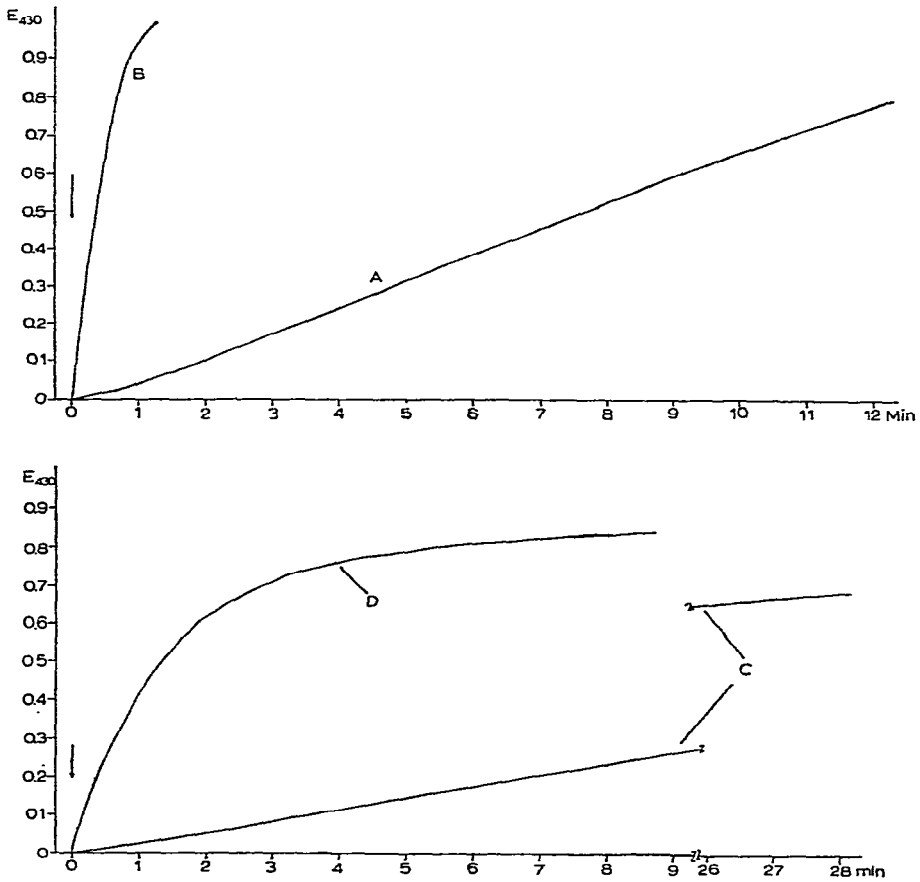


Fig. 8. Enzymic identification of the anomeric configuration of D-glucose liberated by sucrase. Before mutarotation (C) the D-glucose liberated by sucrase is slowly oxidized by the glucose oxidase-peroxidase reagent: after mutarotation, it is oxidized rapidly (D). In A, α-D-glucose, and, in B, β-D-glucose were oxidized under identical conditions (approximately 0.2 μmole in 30 μl of water).

been confirmed recently[17]. Since 1925, however, better preparations of the enzyme have become available, and it has been established that maltotriose, along with maltose, is formed by α-amylase from amylose during the first phase of the hydrolysis[18]. Gas-liquid chromatography of per(trimethylsilyl)ated sugars has been suggested recently for the identification of the anomeric configuration of sugars[6,7]. It seemed appropriate, therefore, to reinvestigate the anomeric configuration of the sugars produced by α-amylase from amylose, with the two-fold purpose of using an independent method to check the anomeric configuration of maltose, and of the

identifying of the anomeric configuration of maltotriose. As expected, we found that both oligosaccharides are released in the α-configuration.

The following criteria were applied for the assessment of the chromatographic peaks to the individual anomers: comparison with chromatograms from authentic samples of crystalline individual anomers (this criterion was not available for maltotriose), comparison with chromatograms from mixtures at mutarotational equilibrium, together with the known quantitative ratios of the anomers and the empirical rule that the retention time increases with the number of equatorial groups[19,20]. In each case, agreement among the criteria allowed unequivocal assessment.

At high α-amylase concentrations, no maltotriose could be detected. The maltose released was in the α-configuration only (Fig. 1, A). At lower α-amylase concentrations, both maltose and maltotriose were detected. They were both clearly released in the α configuration (Fig. 3, bottom). Our results, therefore, confirm and extend Kuhn's conclusions[16], and are compatible with both the double-displacement[21] and the "front-attack"[22] mechanisms suggested for pancreatic α-amylase.

The maltose to maltotriose molar ratio, in the early phase of the experiment described in Fig. 3, was approximately 2.1 (this figure is not very accurate, because maltose and maltotriose were estimated from different chromatograms). This ratio agrees with the one[18] reported by Whelan and Roberts (2.39) and with the ratio calculated for a random attack of amylose by α-amylase (2.35) (see, however, ref. 23). Both the steep increase in the maltose to maltotriose ratio observed at longer incubation times, and the failure of detecting maltotriose in the presence of very large amounts of α-amylase agree with the view that maltotriose itself is also split by pancreatic α-amylase, albeit slowly[24,25].

Barley β-amylase. — By polarimetry this enzyme has been shown to release β-maltose from amylose[26]. Our gas-liquid, chromatographic analysis confirms this conclusion (Fig. 1, B).

Intestinal lactase. — This enzyme, which has been obtained in almost homogeneous state by Wallenfels and Fischer[13] from the calf and by us from the rat[10], had not been previously investigated as to the anomeric configuration of the D-galactopyranose released. Fig. 7 shows that β-D-galactopyranose is liberated by this enzyme from lactose. In fact, β-galactose dehydrogenase from *Pseudomonas fluorescens*, which dehydrogenates β-D-galactopyranose only[8], rapidly oxidized essentially all the D-galactose present in a lactose-lactase mixture after an incubation of 1 min. After attainment of the mutarotation equilibrium, β-galactose dehydrogenase quickly oxidizes approximately two thirds of the D-galactose present, and then slowly the remaining third (Fig. 7, B). The rate-limiting step in the oxidation of this last third of D-galactose is presumably the mutarotation of α-D-galactopyranose (and β-D-galactofuranose) into β-D-galactopyranose. This slow step follows a first order kinetic (Fig. 7, C).

Under the assumption that the reaction involved is $\alpha \overset{k_1}{\rightarrow} \beta$, the velocity constant k_1 was calculated, and found to be 0.88 (on the basis of natural logarithms). It is considerably larger than the coefficient of the spontaneous mutarotation in water

(see below). The difference is probably due to the high ionic strength in the experiment described in Fig. 7 ($\mu \simeq 1.14$), the higher pH value (8.6), the higher temperature (20° versus 15.1°), and the mutarotase activity of high concentrations of 2-amino-2-hydroxymethyl-1,3-propanediol[27].

Gas-liquid, chromatographic analysis of the monosaccharides formed during lactase-catalyzed hydrolysis of lactose (Figs. 4 and 5) again shows that galactose is released as β-D-galactopyranose. In fact, whereas the fraction of β-D-galactopyranose can be extrapolated to 100% at zero time, the fraction of α-D-galactopyranose can be extrapolated to zero, and the so-called γ-galactose (β-D-galactofuranose)[28] never represents more than very few percent, and increases during the course of the reaction (not apparent in the figure).

When we attempted to calculate the fraction of β-D-galactopyranose present at various times of incubation of lactose with lactase (for the equation, see ref. 6), using the velocity constants for spontaneous $\alpha \rightleftarrows \beta$ mutarotation, we constantly observed that the fraction of β-D-galactopyranose found was less than expected. This discrepancy is probably due to a weak galactose-mutarotase activity still present in our lactase preparation (Fig. 6, ▲—▲). The mutarotation coefficient for the reaction α-D-galactopyranose $\rightleftharpoons$ β-D-galactopyranose in water, at 15.1°, is 0.01 min^{-1}. It agrees with the coefficient reported by Bentley *et al.* (ref. 7, from g.l.c.) and by Isbell and Pigman (ref. 4, from polarimetry), consideration being given to the slightly different conditions used, and to the use of decimal, rather than natural logarithms (as here).

Intestinal sucrase. — We have previously produced evidence by g.l.c. that the D-glucose liberated by intestinal sucrase is in the α-configuration[6]. Fig. 8 provides now enzymic evidence that it is so. In fact, the D-glucose liberated is oxidized very slowly (C), unless mutarotation has occurred (D). If these results are compared with those obtained with similar amounts of α-, respectively, β-D-glucose (A, B), it becomes clear that the D-glucose liberated by sucrase is not in the β-configuration.

Mold glucose-oxidase is known to oxidize β-D-glucose preferentially[29], and purified preparations of this enzyme have been used in the determination of mutarotase[27,30]. For the purpose of the present investigation, 0.5mM mercuric chloride was added to the reaction mixture, in order to inhibit[2,5] the traces of mutarotase possibly still present in the enzyme preparations used. It was also ascertained that the glucose-oxidase preparation was essentially free of sucrase activity (less than 1 unit per g of protein).

Intestinal lactase, sucrase, and isomaltase, along with pancreatic α-amylase and a number of other carbohydrases (*e.g.* refs. 8, 21, 31) do not, therefore, produce an inversion at the anomeric carbon of the compound released. Whereas this observation renders unlikely a simple, single S_N2 reaction, it is compatible with both a double displacement and a front attack[22]. The possible participation of an imidazole group to the catalytic action (or to the stabilization of the enzyme molecule) of lactase[13], sucrase[11], and isomaltase[32] is also in agreement with either of these mechanisms.

ACKNOWLEDGMENTS

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SYNTHESIS OF 2-ACETAMIDO-2-DEOXY-3-*O*-β-L-FUCOPYRANOSYL-D-GLUCOSE*

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ABSTRACT

Crystalline 2-acetamido-2-deoxy-3-*O*-β-L-fucopyranosyl-D-glucose was synthesized by condensation of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide with benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside, followed by removal of the protecting groups.

INTRODUCTION

2-Amino-2-deoxy-*O*-α-L-fucopyranosyl-D-glucose disaccharides are part of the determinant-carbohydrate moiety of blood-group substances. A pentasaccharide and a hexasaccharide having Le[a] activity have been shown to contain the 2-acetamido-2-deoxy-4-*O*-α-L-fucopyranosyl-D-glucosyl group[2,3], and 2-acetamido-2-deoxy-6-*O*-α-L-fucopyranosyl-D-glucose was isolated by degradation of a glycoprotein possessing blood-group A activity[4]. More recently, Lloyd *et al.*[5] have described the isolation, from blood-group substances A, B, and H, of an oligosaccharide composed of an L-fucosyl group linked to a 2-acetamido-2-deoxy-D-glucosyl residue at O-3; the anomeric configuration of the L-fucosyl group was not elucidated. In addition, a glycolipid containing a pentasaccharide moiety having a 2-amino-2-deoxy-*O*-α-L-fucopyranosyl-D-glucose residue has been isolated from cancer tissues[6].

The synthesis of 2-acetamido-2-deoxy-3-*O*-β-L-fucopyranosyl-α-D-glucose (**5**) was undertaken as part of a study of disaccharide structures by mass spectroscopy, and of synthetic disaccharide–polypeptide antigens. Occasion was also taken to ascertain whether, under the conditions of the Koenigs–Knorr reaction, 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide[7] (**1**) would be able to form an α-L-glycosidic linkage. Finally, it seemed probable that comparison of the properties of the disac-

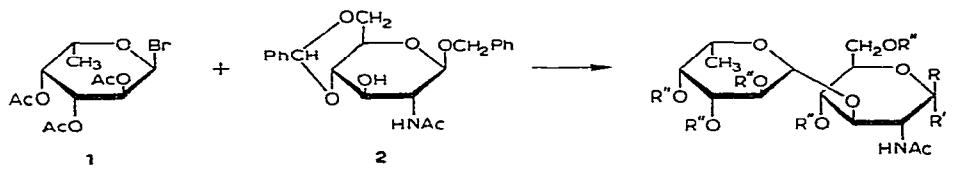
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**On leave of absence from the Weizmann Institute of Science.

charide **5** with those of the product isolated by Lloyd *et al.*[5] might help in the elucidation of the structure of the latter compound.

RESULTS AND DISCUSSIONS

Condensation of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide[7] (**1**) with benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[8] (**2**) was followed by removal of the benzylidene group and acetylation, to give crystalline benzyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl)-β-D-glucopyranoside (**3**) without isolation of the intermediate disaccharides. Removal of the *O*-acetyl groups of the disaccharide **3** gave the crystalline benzyl D-glucoside **4**, the benzyl group of which was hydrogenolyzed to give the desired crystalline 2-acetamido-2-deoxy-3-*O*-β-L-fucopyranosyl-α-D-glucose (**5**). Acetylation of compound **5** gave the crystalline hexaacetate **6**.



3 R = OCH_2Ph; R' = H; R" = Ac
4 R = OCH_2Ph; R' = R" = H
5 R = R" = H; R' = OH
6 R = H; R' = OAc; R" = Ac

Preliminary conclusions, based on the complex n.m.r. spectrum of compound **4**, had led to the erroneous assignment of an α-L-(1→3) linkage to the dissaccharide[1]. However, the β-L configuration of the glycosidic linkage of the dissaccharides **3** to **6** is strongly suggested by a comparison of the optical rotations of the dissaccharides with those of the parent monosaccharides. Although the hypothesis of the additive nature of the relative contribution of each asymmetric center, as exemplified in the isorotation rules, is valid only as a first approximation[9], it gives sufficient information for the determination of the glycosidic linkage of a disaccharide, especially when the value for the corresponding methyl glycosides is used[10-12] (see Table I). The molar rotations of disaccharides composed of a (nonreducing) α- or β-D-galactopyranosyl group linked to various positions of a (reducing) 2-acetamido-2-deoxy-D-glucose residue is in good agreement with the sum of the molar rotations of 2-acetamido-2-deoxy-D-glucose (at equilibrium) and methyl α- or β-D-galactopyranoside, respectively. The (1→3)-linked disaccharide has a molar rotation slightly lower than that of the two other corresponding disaccharides. The molar rotation of the only disaccharide thus far synthesized that contains a (nonreducing) L-fucopyranosyl group, namely, 2-*O*-α-L-fucopyranosyl-D-galactose[17], was also in good agreement with the sum of the molar rotations of methyl α-L-fucopyranoside and D-galactose (at equilibrium). The molar rotation of the (1→3)-linked disaccharide **5** now described is slightly

lower than the sum of the molar rotations of methyl β-L-fucopyranoside and 2-acetamido-2-deoxy-D-glucose (at equilibrium), but is far different from the sum of the molar rotations of methyl α-L-fucopyranoside and 2-acetamido-2-deoxy-D-glucose. Similarly, the molar rotation of the (1→6)-linked disaccharide[18] corresponding to **5** and that of its alditol derivative[18] were in good agreement with the sum of the molar rotations of their respective constituents.

TABLE I

MOLAR ROTATION OF SELECTED DISACCHARIDES COMPARED TO THE SUM OF THE CONSTITUENTS[a]

Compounds	$[M]_D$, *degrees* × 10^{-2}
Methyl α-D-galactopyranoside + 2-acetamido-2-deoxy-D-glucose[b]	+347
2-Acetamido-2-deoxy-6-*O*-α-D-galactopyranosyl-D-glucose[13]	+470
Methyl β-D-galactopyranoside + 2-acetamido-2-deoxy-D-glucose[b]	+ 88
2-Acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-D-glucose[14]	+ 45
2-Acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucose[15]	+112
2-Acetamido-2-deoxy-6-*O*-β-D-galactopyranosyl-D-glucose[16]	+124
Methyl α-D-fucopyranoside + D-galactose[b]	−208
2-*O*-α-L-Fucopyranosyl-D-galactose[17]	−191
Methyl β-L-fucopyranoside + 2-acetamido-2-deoxy-D-glucose[b]	+113
Methyl α-L-fucopyranoside + 2-acetamido-2-deoxy-D-glucose[b]	−264
2-Acetamido-2-deoxy-3-*O*-β-L-fucopyranosyl-D-glucose (**5**)	+ 92
2-Acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-D-glucose[18]	+111
Methyl β-L-fucopyranoside + 2-acetamido-2-deoxy-D-glucitol	0
2-Acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-D-glucitol[18]	− 30

[a]Optical rotation determined for aqueous solutions. [b]At equilibrium.

The α-D configuration of the 2-acetamido-2-deoxy-D-glucose residue of the peracetylated derivative **6** was assumed on the basis of the high specific optical rotation of **6**.

Disaccharides containing an α-D-(1→2) linkage have been synthesized by Lehmann and Beck[19] and Helferich and Zirner[20]. However, investigation of the synthesis of kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) by Koeppen[21] has shown that both the α-D- and the β-D-(1→2) linkages are formed, in the approximate ratio of 1:1. The high proportion of the α-D configuration formed when the linkage is (1→2) is in sharp contrast to the very small proportion of α-D linkage formed when the glycosidic linkage is at O-3, O-4, or O-6 of the reducing hexose moiety.

As Flowers *et al.*[7,17] had synthesized 2-*O*-α-L-fucopyranosyl-D-galactose, it seemed possible that the L-fucopyranosyl group would also exert an influence to cause formation of an α-L-(1→3) linkage; as shown by our results, however, this did not occur.

EXPERIMENTAL

Melting points were determined with a Mettler FP-2 apparatus, and correspond to "corrected melting points". Optical rotations were determined in semimicro tubes

with a Perkin–Elmer Model 141 polarimeter. The chloroform used was A.R. grade and contained approximately 0.75% of ethanol. I.r. spectra were recorded, for potassium bromide discs, with a Perkin–Elmer Model 237 spectrophotometer. N.m.r. spectra were recorded with a Varian A-60 n.m.r. spectrometer. Column chromatography was performed on Silica Gel Davison (grade 950, 60–200 mesh), from the Davison Co., Baltimore, Maryland 21201, which was used without pretreatment. The sequence of eluants was hexane, benzene (or 1,2-dichloroethane), ether, ethyl acetate, acetone, and methanol, individually or in binary mixtures. The ratio of weight of substance to weight of adsorbent was 1:50 to 1:100. The volume of the fractions eluted was 4 ml per g of silica gel. The ratio of diameter to length of the column was 1:20. T.l.c. was performed on Silica Gel G (Kieselgel G, Merck, Darmstadt). Evaporations were conducted *in vacuo*, with the bath temperature kept below 45°. Volumes of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were performed by Dr. M. Manser, Zurich, Switzerland.

Benzyl 2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4-tri-O-acetyl-β-L-fucopyranosyl)-β-D-glucopyranoside (3). — A solution of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[7] (1.5 g) and mercuric cyanide (0.9 g) in 1:1 benzene–nitromethane (180 ml) was concentrated under atmospheric pressure to 130 ml. After the mixture had cooled to room temperature, a solution of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide[7] (1) (2 equiv.) in 1,2-dichloroethane (30 ml) was added, and the mixture was stirred for 3 days at room temperature with exclusion of moisture. The solution was diluted with dichloromethane, washed successively with cold sodium hydrogen carbonate solution and water, dried (sodium sulfate), and evaporated. A solution of the resulting syrup in 60% acetic acid (50 ml) was heated for 30 min at 100°, cooled, and evaporated, and the residue was thoroughly dried in the presence of phosphorus pentaoxide. Acetic anhydride (4 ml) and pyridine (6 ml) were added, and the solution was kept for 24 h at room temperature, and evaporated. The residual syrup was dissolved in benzene, and purified by chromatography on silica gel. A crystalline fraction was obtained by elution with 4:1 ether–acetone. Recrystallization from dichloromethane–ether–hexane gave 1.8 g (64%), m.p. 215–218°, $[\alpha]_D^{20}$ −24° (*c* 1, acetone); ν_{max}^{KBr} 1715 (OAc), and 1660 and 1560 cm^{-1} (NHAc).

Anal. Calc. for $C_{31}H_{41}NO_{15}$: C, 55.75; H, 6.18; N, 2.09; O, 35.98. Found: C, 55.67; H, 6.22; N, 2.18; O, 36.34.

Benzyl 2-acetamido-2-deoxy-3-O-β-L-fucopyranosyl-β-D-glucopyranoside (4). — A solution of compound 3 (1.0 g) in dry methanol (20 ml) containing a catalytic amount of sodium methoxide was kept overnight at 5°. The solution was made neutral by treatment for several min with a small amount of BioRad AG 50W X-16 (H^+) ion-exchange resin, the suspension was filtered, the filtrate was evaporated, and the residue was crystallized from a small volume of cold methanol, to give 0.53 g (81%) of 4, m.p. 277–278°, $[\alpha]_D^{20}$ −48° (*c* 1, methanol); t.l.c. in 4:5:1 butyl alcohol–acetone–water: $R_{lactose}$ 1.48.

Anal. Calc. for $C_{21}H_{31}NO_{10}$: C, 55.14; H, 6.83; N, 3.06; O, 34.97. Found: C, 55.02; H, 6.71; N, 2.87; O, 35.16.

Periodate oxidation of compound **4** was performed by an adaptation of the spectrophotometric method of Aspinall and Ferrier[22]. A sample (14 mg) was dissolved in 10mM sodium metaperiodate solution (10 ml), and this solution and a blank solution were kept in the dark at 20°. Aliquots from both solutions (500 μl) were withdrawn and diluted to 50 ml. The relative absorbance (A) of the two solutions was determined at 223 nm with a Zeiss spectrophotometer. The difference in A_{223} of the two solutions compared with A_{300} (which was taken as the base line) gave a measure of the periodate consumption. A correction was applied for the absorbance of sodium iodate measured under the same conditions (about 13% of that of an equimolar solution of sodium metaperiodate.) The periodate consumption was found to be 1.85 mole per mole of disaccharide, a value in agreement with a (1→3)-linkage.

2-Acetamido-2-deoxy-3-O-β-L-fucopyranosyl-α-D-glucose (**5**). — A solution of compound **4** (200 mg) in a mixture of 95% ethanol (49 ml) and acetic acid (1 ml) was shaken for 24 h at room temperature with 10% palladium-on-charcoal (250 mg) and hydrogen at a pressure of 3 atm. After filtration of the suspension, the filtrate was evaporated to a colorless solid which was recrystallized from cold methanol to give 110 mg (67%) of **5**, m.p. 148–150°, $[\alpha]_D^{20}$ +25→+11° (*c* 0.9, 60% methanol); g.l.c. of the per(trimethylsilyl) derivative, on a 3-m column of 3% OV-17, showed a major peak at t'_R 26.0 (relative to hexa-*O*-(trimethylsilyl)-*myo*-inositol[23]), followed by a very minor peak at t'_R 27.2 (probably the β-D anomer).

Anal. Calc. for $C_{14}H_{25}NO_{10}$: C, 45.77; H, 6.86; N, 3.81; O, 43.55. Found: C, 45.98; H, 6.92; N, 3.56; O, 42.95.

Acetylation with acetic anhydride and pyridine at room temperature gave 2-acetamido-1,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-(2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl)-α-D-glucose (**6**), which crystallized from acetone–hexane; m.p. 232–234°, $[\alpha]_D^{20}$ +61° (*c* 1, chloroform).

Anal. Calc. for $C_{26}H_{37}NO_{16}$: C, 50.39; H, 6.02; N, 2.25; O, 41.31. Found: C, 49.69; H, 6.03; N, 2.16; O, 40.56.

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SYNTHESIS OF 2-ACETAMIDO-2-DEOXY-6-*O*-β-L-FUCOPYRANOSYL-D-GLUCOSE*

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ABSTRACT

Crystalline 2-acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-D-glucose was synthesized by condensation of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide either with benzyl 2-acetamido-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside or with benzyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside, followed by removal of the protecting acetyl and benzyl groups. The resulting disaccharide was characterized by means of a crystalline alditol derivative.

INTRODUCTION

In the preceding communication[1], the synthesis of 2-acetamido-2-deoxy-3-*O*-β-L-fucopyranosyl-D-glucose was described as part of a study of disaccharide structures by mass spectroscopy and of synthetic disaccharide–polypeptide antigens[1]. The present paper describes the synthesis of the (1→6) analog, namely, 2-acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-D-glucose (**9**).

RESULTS AND DISCUSSION

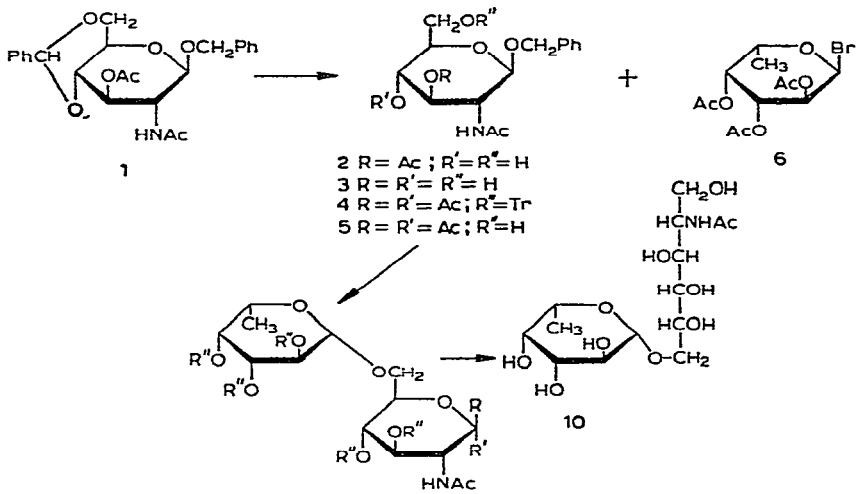
Attempts to obtain a disaccharide having a (reducing) 2-acetamido-2-deoxy-D-glucose residue linked at O-4 have shown the lack of reactivity at this position on a pyranose ring; condensation at O-4 took place only when the acyclic form was present[2]. Consequently, in order to obtain a (1→6)-linked disaccharide, protection of the hydroxyl group on C-4 of benzyl 2-acetamido-3-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**2**) was not necessary. The starting material **2**, obtained from the 4,6-benzylidene acetal[3] **1**, was condensed directly with 2,3,4-tri-*O*-acetyl-L-fucopyranosyl bromide[4] (**6**) in the presence of mercuric cyanide[5]. The resulting disacchar-

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**On leave of absence from the Weizmann Institute of Science.

ide was deacetylated to give the crystalline compound **8**, which was found to be identical with the product described next.

In order to obtain the disaccharide **8** by an unambiguous route, benzyl 2-acetamido-3,4-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**5**) was prepared from the known benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside[6] (**3**) through the 6-*O*-trityl intermediate **4**, and D-glucoside **5** was condensed with the bromide **6**. The resulting, fully acetylated, crystalline disaccharide (mainly **7**) was shown by thin-layer chromatography to contain a small proportion of the α-L-linked disaccharide. Chromatography of benzyl 2-acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-β-D-glucopyranoside (**8**), resulting from alkaline saponification of **7**, gave a pure product, which was then hydrogenolyzed to give crystalline 2-acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-α-D-glucopyranose (**9**).



It is of interest that, in this case also, the condensation of the bromide **6** in presence of mercuric cyanide gave mainly the β-L-linked disaccharide; the formation of some α-L-linked disaccharide is indicated by the low optical rotation of the impure disaccharide **8**. The anomeric configuration of the disaccharide **9**, and, consequently, of the parent disaccharides **7** and **8**, was deduced by the approximation described in the preceding article[1]. Addition of the molar optical rotation of methyl β-L-fucopyranoside (+2,500) to that of 2-acetamido-2-deoxy-D-glucose at equilibrium (+8,800) gave a total of +11,300, as compared to a $[M]_D$ value of +11,100 for **9**. For an α-L-linked disaccharide, the calculated sum (on the basis of $[M]_D$ −35,200 for methyl α-L-fucopyranoside) would be −26,400 (see Table I in ref. 1).

The disaccharide **9** gave an amorphous peracetate, and, on reduction with sodium borohydride, very hygroscopic, crystalline 2-acetamido-2-deoxy-6-*O*-β-L-fucopyranosyl-D-glucitol (**10**). The molar rotation of compound **10** was −3,000,

in good agreement with the sum (0) of the molar rotations of methyl β-L-fucopyranoside and 2-acetamido-2-deoxy-D-glucitol, and very different from the sum (−38,000) of the molar rotations of methyl α-L-fucopyranoside and 2-acetamido-2-deoxy-D-glucitol. Thus, the β-L-(1→6) linkage of the synthesized disaccharide **9** has been well established.

EXPERIMENTAL*

Benzyl 2-acetamido-3-O-acetyl-2-deoxy-β-D-glucopyranoside (**2**). — A solution of benzyl 2-acetamido-3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside[3] (**1**) (800 mg) in 60% acetic acid (10 ml) was heated for 0.5 h at 100°, and evaporated, and toluene was several times added to and evaporated from the residue to remove traces of acetic acid. The residue was crystallized from methanol–ether, to give 500 mg (79%) of **2**, m.p. 191–193° (sintering at 186°), $[\alpha]_D^{20}$ −79° (*c* 1.0, methanol); ν_{max}^{KBr} 3400–3500 (OH), 3350 (NH), 1715 (OAc) and 1660, 1560 cm^{-1} (secondary amide).

Anal. Calc. for $C_{17}H_{23}NO_7$: C, 57.78; H, 6.56; N, 3.96; O, 31.69. Found: C, 57.69; H, 6.62; N, 3.93; O, 31.85.

Benzyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-6-O-trityl-β-D-glucopyranoside (**4**). — A suspension of benzyl 2-acetamido-2-deoxy-β-D-glucopyranoside[6] (**3**; 2.1 g) and chlorotriphenylmethane (1.9 g) in dry pyridine (15 ml) was shaken at room temperature until a clear solution had been obtained. After 2 days, acetic anhydride (5 ml) was added, with cooling. The solution was kept for another 2 days at room temperature, and then poured into ice–water (500 ml). The precipitate was filtered off, washed with water, dried (4.2 g, 95%), and recrystallized from chloroform–hexane, m.p. 206–207°; $[\alpha]_D^{20}$ −104° (*c* 1.0, chloroform).

Anal. Calc. for $C_{38}H_{39}NO_8$: C, 71.57; H, 6.16; N, 2.20; O, 20.07. Found: C, 71.40; H, 6.10; N, 2.33; O, 20.28.

Benzyl 2-acetamido-3,4-di-O-acetyl-2-deoxy-β-D-glucopyranoside (**5**). — A solution of **4** (3 g) in glacial acetic acid (15 ml) was cooled to 15°, and a 32% solution of hydrogen bromide in glacial acetic acid (1.3 ml) was added. The mixture was shaken for 75 sec, and then quickly filtered. The filtrate was immediately poured into ice–water (70 ml), and the bromotriphenylmethane on the sintered-glass filter was washed with cold water (80 ml). The filtrate and washings were combined, and extracted several times with chloroform, and the combined extracts were evaporated without further treatment. The residue was freed from traces of acetic acid by repeated addition of toluene followed by evaporation, and was then crystallized from chloroform–hexane to give 1.3 g (85%) of **5**, m.p. 176–177°; $[\alpha]_D^{20}$ −28° (*c* 1, ethanol).

Anal. Calc. for $C_{19}H_{25}NO_8$: C, 57.71; H, 6.37; N, 3.54; O, 32.37. Found: C, 57.61; H, 6.28; N, 3.61; O, 32.29.

*See Ref. 1.

Benzyl 2-acetamido-2-deoxy-6-O-β-L-fucopyranosyl-β-D-glucopyranoside (**8**). *A. — From* **5**. A solution of **5** (1.24 g) and mercuric cyanide (900 mg) in 1:1 benzene–nitromethane (160 ml) was distilled at atmospheric pressure until the volume was 130 ml, and cooled to room temperature. A solution of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide[4] (**6**) (1.8 g) in 1,2-dichloroethane (30 ml) was added, and the mixture was stirred for 3 days at room temperature. Additional amounts of bromide **6** (1 g) and mercuric cyanide (500 mg) were then added, and the mixture was stirred for 24 h. After being cooled, the mixture was diluted with chloroform (50 ml), washed successively with a cold, saturated solution of sodium hydrogen carbonate and water, dried (sodium sulfate), and evaporated. The residue was dissolved in chloroform, and chromatographed on a column of silica gel. Elution with 3:1 ether–acetone gave a fraction enriched in disaccharide **7**. Crystallization of this fraction from benzene–ether gave a product (240 mg) having m.p. 98–100°, $[\alpha]_D^{20}$ −38° (*c* 0.5, methanol), which, by t.l.c. (in ethyl acetate), showed one major spot and one minor spot; the latter migrated slightly the faster and was probably the α-L anomer of **7**.

Anal. Calc. for $C_{31}H_{41}NO_{15}$: C, 55.75; H, 6.18; N, 2.09. Found: C, 56.17; H, 6.11; N, 2.36.

As alkaline hydrolysis of this product gave an impure disaccharide, the crude, acetylated product obtained by evaporation of the chloroform extracts was dissolved in dry methanol (30 ml) containing a catalytic amount of sodium methoxide, and the solution was kept for 24 h at 0°. After removal of the sodium ions by treatment with BioRad AG-50W X-16 (H^+) ion-exchange resin, the resin was filtered off, and the filtrate was evaporated. The residue was dissolved in 2:1 benzene–methanol, and the solution was chromatographed on silica gel E. Merck AG (70–325 mesh, ASTM). Elution with 2:1 benzene–methanol gave almost pure compound **8** (730 mg, 39%), m.p. 232–234°; recrystallization from ethanol raised the m.p. to 237–239°. The compound had $[\alpha]_D^{20}$ −37° (*c* 1, methanol); n.m.r. data (deuterium oxide); τ 2.73 (5 H, Ph); τ 8.1 (*N*-Ac); and τ 8.8, doublet, *J* 6.5Hz (3 H, Me). On oxidation with periodate[7], **8** consumed 2.9 moles per mole during 72 h at 20°.

Anal. Calc. for $C_{21}H_{31}NO_{10}$: C, 55.14; H, 6.83; N, 3.06; O, 34.97. Found: C, 55.17; H, 6.85; N, 2.91; O, 34.92.

B. From **2**. The mixture of compound **2** and 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide (**6**) was treated in the way just described for compound **5**. After removal of the *O*-acetyl groups, examination, by t.l.c. in two solvent-systems, of the reaction products indicated the formation of **8** and no other disaccharide could be detected; t.l.c. data: on cellulose, with 4:5:1 butyl alcohol–acetone–water, R_3 0.35; on silica gel G, with 4:5:1 butyl alcohol–acetone–water, R_3 0.70, and with 2:1 benzene–methanol, R_3 0.60.

2-Acetamido-2-deoxy-6-O-β-L-fucopyranosyl-α-D-glucopyranose (**9**). — A solution of **8** (200 mg) in 95% ethanol (150 ml) containing acetic acid (2 ml) was shaken in the presence of 10% palladium-on-charcoal (150 mg) for 48 h at room temperature and 3 atm. The suspension was filtered, and the filtrate was evaporated to a syrup

which was crystallized from methanol–acetone to give a very hygroscopic material (120 mg, 73%) having m.p. 144–146°, $[\alpha]_D^{20}$ +31→29° (*c* 0.8, 70% ethanol); t.l.c. data: on silica gel G in 4:5:1 butyl alcohol–acetone–water, R_3 0.43.

Anal. Calc. for $C_{14}H_{25}NO_{10}$: C, 45.77; H, 6.86; N, 3.81; O, 43.55. Found: C, 45.44; H, 6.90; N, 3.48; O, 43.23.

The mother liquor from the crystallization of **9** was evaporated to dryness, and the residue was acetylated with pyridine–acetic anhydride to give an amorphous material, $[\alpha]_D^{20}$ +37° (*c* 1.3, chloroform).

Anal. Calc. for $C_{26}H_{37}NO_{16}$: C, 50.39; H, 6.02; N, 2.25; O, 41.34. Found: C, 50.21; H, 6.04; N, 2.37; O, 41.20.

2-Acetamido-2-deoxy-6-O-β-L-fucopyranosyl-D-glucitol (**10**). — To a solution of **9** (100 mg) in water (5 ml), sodium borohydride (50 mg) was added, and the mixture was kept for 24 h at room temperature. Acetic acid (a few drops) was added, and the solution was immediately passed through a column of Dowex-50 AG (50W X-16) (H^+) ion-exchange resin. The aqueous eluate was evaporated, and several additions of ethanol, followed by evaporation, were made (to dry the crystalline residue). It was difficult to keep the product crystalline in the presence of air, because of its extreme hygroscopicity, $[\alpha]_D^{20}$ −8° (*c* 0.6, methanol); t.l.c. data: on silica gel G, with 4:5:1 butyl alcohol–acetone–water, R_3 0.32. Hydrolysis of **10** with M hydrochloric acid for 1 h at 100° gave a product that, on t.l.c., showed a spot corresponding to fucose and a spot corresponding to 2-acetamido-2-deoxy-glucitol.

Anal. Calc. for $C_{14}H_{27}NO_{10}\cdot H_2O$: C, 43.41; H, 7.55; N, 3.62. Found: C, 43.25; H, 7.41; N, 3.28.

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PREPARATION OF ALKYL α-D-GLUCOPYRANOSIDES

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ABSTRACT

An improved preparation of alkyl α-D-glucopyranosides involves (*a*) reaction of β-D-glucopyranose pentaacetate or a similar ester with an alcohol in the presence of an acid catalyst until equilibrium is attained, (*b*) hydrolysis of the alkyl β-D-glucopyranoside present in the reaction mixture with a β-D-glucosidase, and (*c*) removal of the D-glucose and the alcohol formed by this hydrolysis. This sequence gave twelve pure alkyl α-D-glucopyranosides, each in high yield. The following alkyl α-D-glucopyranosides are reported for the first time (in unacetylated form): 2-propyl, 2-butyl, 2-methylpropyl, 1-pentyl, and 2,2-dimethylpropyl.

INTRODUCTION

The synthesis of alkyl α-D-glucopyranosides is generally more difficult than that of the corresponding β-D anomers. Several methods have been reported for the preparation of α-D-glucopyranosides; most of them involve several steps, with a low overall yield of the α-D-glucoside, contaminated with the β-D-glucoside.

Fischer[1] prepared α-D-glucopyranosides by refluxing a solution of D-glucose in a lower alcohol containing an acid catalyst. Bourquelot and Verdon[2] synthesized α-D-glucosides through the action of α-D-glucosidase on a dilute solution of D-glucose in an alcohol. Fischer and Bergmann[3] modified the Koenigs–Knorr reaction by neutralizing the hydrogen chloride with quinoline in order to prepare a mixture of α- and β-D-glucopyranosides. A mixture of the anomers is also formed when mercury(II) salts[4] are used in the Koenigs–Knorr reaction.

Pacsu[5] treated dithioacetals of monosaccharides with mercuric chloride in the appropriate alcohol to give a mixture of glucosides (50–90% yield) containing about 70% of the α-D anomer. Pacsu[6,7] also found that a β-D-glucoside tetraacetate, prepared by the Koenigs–Knorr reaction, could be anomerized with a Lewis acid catalyst, such as titanium tetrachloride or boron trifluoride, in anhydrous chloroform. Alcoholysis of tetra-*O*-acetyl-β-D-glucopyranosyl chloride in the presence of silver

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TABLE I

YIELDS AND PROPERTIES OF ALKYL α- AND β-D-GLUCOPYRANOSIDES FROM D-GLUCOPYRANOSE PENTAACETATE

α,β-D-*Glucopyranoside*	*Reaction temperature (degrees)*	*Yield*[a] (g)	*Yield*, %	α-D *Anomer*		β-D *Anomer*	
				R_T (*min*)[b]	%	R_T (*min*)[b]	%
Methyl	57	12.9	100	10.9	75	12.0	25
Ethyl	71	13.4	97	10.7	77	13.7	23
Allyl	78	14.4	99	13.9	74	17.1	26
1-Propyl	76	14.7	100	13.4	78	19.1	22
2-Propyl	74	14.7	100	10.9	80	15.0	20
1-Butyl	81	15.7	100	18.3	85	26.4	15
2-Butyl	74	13.5	86	14.8	76	20.8	24
2-Methylpropyl	75	15.7	100	15.3	77	21.6	23
1-Pentyl	84	16.0	97	26.2	83	38.4	17
2,2-Dimethylpropyl	65	7.2	41	15.7	75	22.8	25
Cyclohexyl	83	16.8	96	45.8	73	67.5	27
Benzyl	85	12.0	76	76.2	79	109.6	21

[a]From 26 g of D-glucopyranose pentaacetate. [b]In g.l.c.

salts has been used for preparing alkyl α,β-D-glucopyranosides in the anomeric ratio of 2:1.

Helferich and Johannis[9] treated 1-*O*-(2,4,6-trimethylbenzoyl)-β-D-glucopyranose with an alcohol in the presence of an acid catalyst to obtain an anomeric mixture of alkyl D-glucopyranosides. Hydrolysis of this mixture with emulsin (crude β-D-glucopyranosidase; β-D-glucoside glucohydrolase, 3.2.1.21) cleaved the β-D-glucoside to D-glucose, which was removed as the (*p*-tolylsulfonyl)hydrazone; pure alkyl α-D-glucopyranosides were isolated in yields of ~50%. Wolfrom and Gilliam[10] prepared methyl α-D-glucopyranoside in 25% yield by the displacement of the 1-*O*-nitro group from β-D-glucopyranose pentanitrate in boiling methanol containing silver carbonate. Lemieux *et al.*[11] formed α-D-glucopyranosides in high yields by deoximation of 3,4,6-tri-*O*-acetyl-2-deoxy-2-oximino-α-D-*arabino*-hexopyranosides, followed by borohydride reduction of the 2-hexulosides so produced. Finally, α,β-D-glucopyranosides have been prepared by trans-D-glucosidation from methyl α-D-glucopyranoside[12] and from α-D-linked polysaccharides[13].

The present report describes the preparation of alkyl α-D-glucopyranosides in high yield and purity by a procedure similar to that used by Helferich and Schmitz-Hillebrecht[14] for preparing aryl α-D-glucopyranosides.

DISCUSSION

Several acids were used as catalysts for the alcoholysis of β-D-glucopyranose pentaacetate. Titratable free acid was lost with hydrogen chloride and sulfuric acid, due to volatilization and esterification, respectively, but not with methanesulfonic acid[9] or *p*-toluenesulfonic acid[15]. All results presented here were obtained with *p*-toluenesulfonic acid monohydrate. Each of the catalysts tried [including Amberlite IR-120 (H^+) cation-exchange resin**, aluminium chloride, and zinc chloride] gave, after 72 h of reaction, an equilibrium mixture that contained at least 73% of the α-D-glucopyranoside (see Table I).

Analysis, by t.l.c. and g.l.c., of the reaction mixture at early stages showed that a major component (50–65%) had formed after 1–2 h. After 5–6 h, this compound is largely converted into alkyl α-D-glucopyranoside. The maximum proportion of the unknown component depends on the alcohol used. When methanol was used, the percentage after 2 h of reaction was 65%; with ethanol, 50%; and with propyl alcohol, 0%. It was noted (t.l.c.) that deacetylation decreases in the same order.

It has been known for many years that furanosides, as well as pyranosides, are formed during acid-catalyzed alcoholysis of sugars[16]. In addition, acyclic acetals and hemiacetals are possible reaction-products or intermediates. Although acyclic acetals were first proposed[1] as likely precursors of glycosides in 1893, they escaped detection for almost 75 years[17]. Ferrier and Hatton[18] found acetals in the mixture obtained by glycosidation of D-glucose, but the maximum concentration was only 2.5%.

**A product of Rohm and Haas Co., Inc., Philadelphia, Pennsylvania.

Identification of the component detected by g.l.c. failed, because we found that D-glucose dimethyl acetal, methyl D-glucofuranoside, and the unknown component all have the same R_F (0.56) in t.l.c. and the same R_T (6.2 min) in g.l.c. with the conditions we used. However, as the rate of deacetylation is proportional to the amount of this component present, it is suggested that the compound is a furanoside[19], because, for the furanoside to be formed, O-4 would first have to be deacetylated. Evidence that this is, indeed, the case was obtained from a comparable methanolysis of β-D-glucopyranose pentabenzoate. The (more stable) benzoic ester groups were removed much more slowly, and none of the unknown component was formed; nevertheless, the formation of methyl α,β-D-glucopyranoside occurred at about the same rate as with β-D-glucopyranose pentaacetate. Replacement of acetyl groups by benzoyl groups should have had little effect on formation of a dimethyl acetal. In addition, this evidence suggests that the unknown component, believed to be a furanoside, is not an intermediate in the formation of the pyranoside.

Complete deacetylation occurred during the alcoholysis. Considerable formation of oligosaccharide was observed when 2,2-dimethylpropanol was used: reversion occurs in this almost anhydrous system, in the presence of acid, before the bulky 2,2-dimethylpropyl group can be incorporated. Replacement of the β-D-glucopyranose pentaacetate by methyl α-D-glucopyranoside tetraacetate, methyl α-D-glucopyranoside, amylose triacetate, or cellulose triacetate gave the same mixture of D-glucopyranosides in the ratio of α:β of approximately 3:1. With the polysaccharides, however, the product was contaminated with oligosaccharides.

The alkyl β-D-glucopyranosides present were completely removed from the reaction mixture by emulsin-catalyzed hydrolysis[9]. The resulting D-glucose was removed by passing the mixture of alkyl α-D-glucopyranoside and D-glucose through a column of Amberlite IRA-401S (OH^-) ion-exchange resin**. All of the D-glucose was retained on the column, whereas the α-D-glucopyranoside was completely eluted with water. Two other methods for removal of D-glucose are equally effective, namely, adsorption of the alkyl α-D-glucopyranoside on carbon, and oxidation of D-glucose to D-gluconolactone, but are, perhaps, not so convenient. In the first of these methods, D-glucose was removed from the carbon by elution with water followed by 1% ethanol; then the alkyl α-D-glucopyranoside was eluted with 50% ethanol. In the second method, the D-glucose was oxidized with D-glucose oxidase (D-glucose: O_2 oxidoreductase, 1.1.3.4). However, since D-glucono-1,5-lactone is a powerful, specific, competitive inhibitor of β-D-glucosidase[20], the two enzymes must be used successively, not concurrently. Alternatively, the D-glucose can be oxidized with bromine water[21]. After either oxidation, D-gluconolactone was removed by (*a*) conversion into barium D-gluconate by heating with a saturated solution of barium hydroxide, (*b*) neutralization with carbon dioxide, (*c*) removal of most of the barium carbonate and barium D-gluconate by filtration, (*d*) evaporation of the filtrate to a syrup, and (*e*) extraction of the α-D-glucopyranoside with 70% ethanol.

The α-D-glucopyranosides are deliquescent. Because of this property, and because they have an axially attached alkyl group, crystal formation is difficult.

However, all were crystallized from ether or acetone. Some of their physical properties are given in Table II.

EXPERIMENTAL

General. — Melting points were obtained on a Thomas–Hoover capillary melting-point apparatus (Uni-Melt), and are uncorrected. Optical rotations were obtained at room temperature (33°) with a Bendix ETL-NPL automatic polarimeter equipped with a sodium (589 nm) interference filter; 1.5% aqueous solutions were used.

T.l.c. was accomplished on Silica Gel H* with an irrigant[34] of 9:6:3:1 (v/v) butyl alcohol–acetic acid–ether–water.

G.l.c. was performed on a Varian Aerograph Model 204-B gas chromatograph with a flame-ionization detector. Per(trimethylsilyl) (TMS) derivatives were prepared by the method of Brobst and Lott[35], and were injected into a stainless-steel column (6 ft by 0.25 in i.d.) packed with 5% SE-30 silicone gum rubber (methyl) on acid-washed, 60–80 mesh, dichlorodimethylsilane-treated Chromosorb W. The column temperature was 160°, and the injection port and detector temperatures were 300°. The carrier gas was helium at a flow rate of 35 ml/min and an inlet pressure of 40 lb. in^{-2}. Hydrogen and air were supplied to the detector at a flow rate of 15 ml/min (inlet pressure, 5 lb. in^{-2}) and 300 ml/min (inlet pressure, 3 lb. in^{-2}), respectively.

Alcoholysis. — β-D-Glucopyranose pentaacetate[36] (26 g) and 200 ml of chloroform were placed in a 1-liter, round-bottomed flask. A solution of *p*-toluenesulfonic acid monohydrate (5.3 g) in the selected alcohol (250 ml) was added, and the mixture was boiled for 72 h under reflux; the reflux temperatures are recorded in Table I. The solution was then cooled to room temperature, and made neutral with Amberlite IR-45 (OH^-) ion-exchange resin**. After filtration and several washings of the resin with aqueous alcohol, the filtrate and washings were combined, and evaporated under diminished pressure (benzyl alcohol was removed by steam distillation); the yield was, in most cases, quantitative. If colored, the syrup in 95% ethanol was stirred with Darco G-60 carbon, the suspension was filtered, and the filtrate was evaporated. The resulting syrup was analyzed by t.l.c. and g.l.c. (see Table I).

Hydrolysis with emulsin. — The mixture of D-glucosides was dissolved in 400 ml of acetate buffer (pH 5; 0.1M) and 0.2 g (760 units) of emulsin in 100 ml of water was added. This mixture was kept for 2 days at 37°, and then treated with a mixture of Amberlite IR-45 (OH^-) and IR-120 (H^+) ion-exchange resins**. After filtration, and several washings of the resins with aqueous alcohol, the filtrate and washings were combined, and evaporated under diminished pressure; the calculated yield was always obtained. Analysis by g.l.c. showed complete removal of the β-D anomer and the formation of D-glucose.

*A product of Brinkmann Instruments Co., Westbury, L. I., New York.

TABLE II

SOME PHYSICAL CONSTANTS OF ALKYL α-D-GLUCOPYRANOSIDES

α-D-*Glucopyranoside*	*Crystallization solvent*	*Melting point (degrees)*		R_F (*t.l.c.*)	$[\alpha]_D^{33}$ (*degrees*)		*References*
		Found[a]	*Reported*		*Found*	*Reported*	
Methyl	ethanol	166–167	168	0.50	+160	+158	22
Ethyl	acetone	113–114	113—114	0.60	+153	+152	23–26
Allyl	ether	99–101.5	100.5–101.5	0.67	+149	+151	27,28
1-Propyl	acetone	89–90	89–90	0.68	+146	+142	27,29
2-Propyl	ether	111–112		0.65	+146		
1-Butyl	ether	80–82	86–87	0.72	+134	+135	11
2-Butyl	ether	74–76		0.71	+144		
2-Methylpropyl	acetone	109–110		0.73	+134		
1-Pentyl	ether	82–83		0.77	+133		
2,2-Dimethylpropyl	ether	124–125		0.78	+131		
Cyclohexyl	ether	125	126	0.74	+136	+137	30,31
Benzyl	ether	121–122	122	0.74	+133	+133	32,33

[a]Uncorrected.

Treatment with D-*glucose oxidase.* — After the β-D-glucopyranoside had been completely removed, D-glucose oxidase (0.2 g; 2,600 units) in 100 ml of water was added to the mixture, and oxygen or air was bubbled continuously through the system for two days. Paper chromatography [irrigant: 9:5:7 (v/v) butyl alcohol–pyridine–water] was used for determining when conversion of the D-glucose into D-gluconolactone was complete. Processing of the mixture was the same as that used after the treatment with emulsin.

Removal of D-*gluconolactone.* — The mixture of alkyl α-D-glucopyranoside and D-gluconolactone was treated with a saturated solution of barium hydroxide solution (600 ml) for 12 h on a steam bath. The volume was readjusted to 600 ml, and Dry Ice was added to bring the pH of the reaction mixture to 8. After filtration of the suspension, the filtrate was evaporated; the resulting syrup was extracted with 70% ethanol, and the extract was treated with Amberlite IR-120 (H^+) ion-exchange resin** to remove any barium ions remaining. Filtration and evaporation gave pure alkyl α-D-glucopyranoside as a clear syrup (overall yield, ~75%).

Removal of D-*glucose.* — The syrup from the emulsin treatment (containing a mixture of the α-D-glucoside and D-glucose) was dissolved in 50 ml of water and placed on a column (50 × 6 cm) of Amberlite IRA-401S (OH^-) anion-exchange resin**. After 12 h on the column, the pure α-D-glucopyranoside was eluted with water; the yield was that calculated, and the D-glucose remained bound to the resin. The first 500–1,000 ml of column eluate was basic, and was made neutral with Amberlite IR-120 (H^+) cation-exchange resin** before evaporation.

Crystallization and analysis. — The syrupy α-D-glucopyranosides were crystallized from ether or acetone, to yield colorless, hygroscopic, crystalline powders. Some of their physical constants are recorded in Table II. The elemental analyses for those not previously reported are given.

2-Propyl α-D-*glucopyranoside.* — *Anal.* Calc. for $C_9H_{18}O_6$: C, 48.65; H, 8.18. Found: C, 48.55; H, 8.11.

2-Butyl α-D-*glucopyranoside.* — *Anal.* Calc. for $C_{10}H_{20}O_6$: C, 50.85; H, 8.53. Found: C, 50.62; H, 8.51.

2-Methylpropyl α-D-*glucopyranoside.* — *Anal.* Calc. for $C_{10}H_{20}O_6$: C, 50.85; H, 8.53. Found: C, 50.56; H, 8.33.

1-Pentyl α-D-*glucopyranoside.* — *Anal.* Calc. for $C_{11}H_{22}O_6$: C, 52.80; H, 8.86. Found: C, 52.60; H, 8.85.

2,2-Dimethylpropyl α-D-*glucopyranoside.* — *Anal.* Calc. for $C_{11}H_{22}O_6$: C, 52.80; H, 8.86. Found: C, 52.51; H, 8.69.

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Note

Unsymmetrical dithiobis(thioformates)

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Previously, we showed that derivatives containing dithiobis(thioformate) groups* undergo rearrangement–fragmentation** under the proper conditions to give thionocarbonates[1] or *S*-alkyl dithiocarbonates[2]. In these studies[1,2], symmetrical derivatives were prepared by crosslinking the corresponding sodium dithiocarbonate (xanthate) derivative with iodine solution. The current objective was to prepare and investigate the behavior of sugar derivatives of the type RO(C=S)SS(C=S)OR′, where R is a sugar moiety and R′ is an alkyl group. Such unsymmetrical sugar derivatives have not been reported to date.

When a solution of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-(sodium dithiocarbonate) (**1**) was mixed with an equimolar amount of sodium ethylxanthate **2**, and iodine solution was added, bis(1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose) 3,3′-[dithiobis(thioformate)] (**3**) and *O*,*O*′-diethyl dithiobis(thioformate) (**4**) were the only major products detectable on t.l.c. As the amount of compound **2** added was increased to about three parts, a major spot appeared that had an R_F value slightly higher than that of **3**; this compound was isolated, and was identified as 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-[*O*-ethyl dithiobis(thioformate)] (**5**) by elemental analysis and u.v. spectrum.

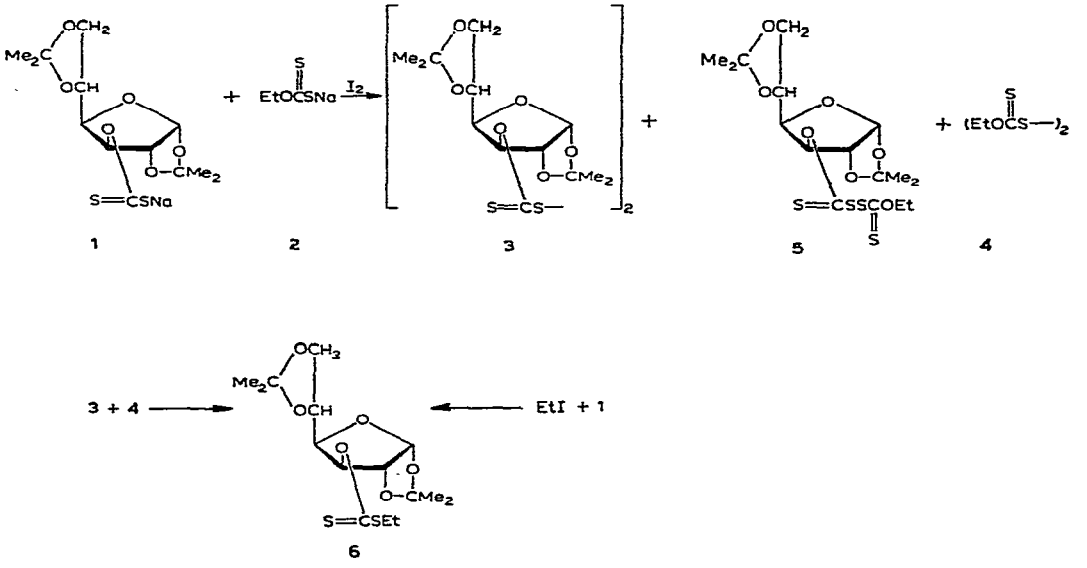
The unsymmetrical dithiobis(thioformate) (**5**) was also formed when a tetrahydrofuran (THF) solution of **3** was mixed with **4** (2:11 molar ratio) and kept at 25°. During 18 h, almost 70% of **3** was converted into **5**, and, after 4 days, the conversion was greater than 90%. The formation of **5** was more rapid if the starting mixture was dissolved in THF containing 1% of pyridine. In this system, about 90% conversion of **3** into **5** occurred in 4 h.

When the solution in THF–pyridine was kept overnight, crystals of sulfur were deposited. T.l.c. of the reaction mixture revealed a single sugar component having an R_F value similar to that of **5**. This product was isolated by preparative t.l.c.; its u.v. spectrum did not show the absorptions characteristic of dithiobis-

*Nomenclature recommended by Chemical Abstracts. In earlier reports, these were called bis-(*O*-thiocarbonyl) disulfide groups.

**For the rearrangement of dithiobis(thioformate) derivatives located on the anomeric carbon atoms, see ref. 1d.

(thioformate) (absorption maxima at 240 and 280 nm, with a peak ratio of about 2:1), but showed absorptions for *S*-alkyl dithiocarbonate (maxima[2] at 225 and 285 nm, with a peak ratio of about 1:2). The sugar component was identified as 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-(*S*-ethyl dithiocarbonate) (**6**) by elemental analysis and by its independent synthesis from **1** and iodoethane.



Scheme 1

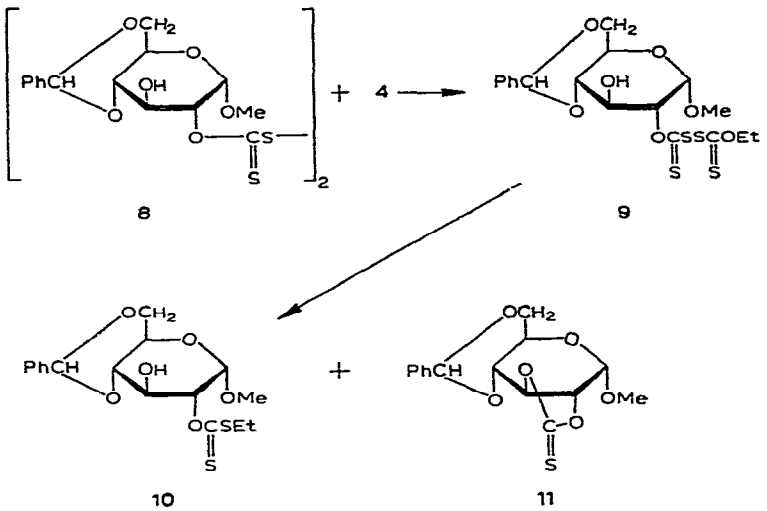
The conversion of **3** into **6** was complete in 4 h when 30% pyridine in THF was used, and was complete immediately if **3** was dissolved in pyridine and treated with **4** at 95°.

The unsymmetrical dithiobis(thioformate) (**5**) was unstable and disproportionated slowly on being kept, to give a mixture of **3**, **4**, and **5**. A similar mixture formed rapidly upon dissolution of **5** in pyridine and, after several h, **6** was the only carbohydrate material present.

Treatment of **3** in THF at 25° in the presence of pyridine with *O*,*O*-dimethyl dithiobis(thioformate) (instead of the ethyl, analog) gave none of the expected, unsymmetrical dithiobis(thioformate), and the corresponding *S*-methyl dithiocarbonate (**7**) was obtained. Repetition of the treatment, but without pyridine, gave the unsymmetrical derivative, which disproportionated like **5**. It is noteworthy that *O*,*O'*-dimethyl dithiobis(thioformate) decomposes almost instantly on contact with pyridine, to give *O*,*S*-dimethyl dithiocarbonate (λ_{max}^{EtOH} 278 nm, ε 11,700, and 221 nm, ε 6,650), carbonyl sulfide, and elemental sulfur. *O*,*S*-Dimethyl dithiocarbonate is not the reactive species involved in the formation of **7**, since no reaction occurs when *O*,*S*-dimethyl dithiocarbonate is added to **3** in the presence of pyridine at 25°. Apparently, in the first step, unsymmetrical dithiobis(thioformate) forms, and this,

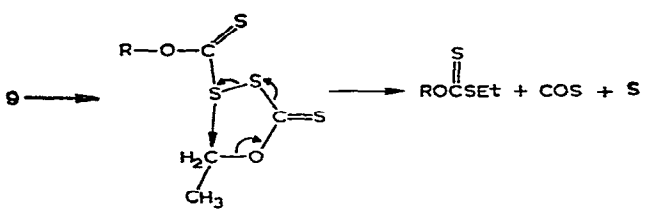
in turn, reacts further, to give the corresponding *S*-methyl dithiocarbonate derivative of the sugar.

Bis(methyl 4,6-*O*-benzylidene-α-D-glucopyranoside) 2,2'-[dithiobis(thioformate)] (**8**) reacted with **4**, both in THF and in THF–pyridine, to give the corresponding, unsymmetrical dithiobis(thioformate) (**9**). In THF–pyridine, further reaction occurred readily, to yield the dithiocarbonic ester (**10**) and methyl 4,6-*O*-benzylidene-α-D-



glucopyranoside 2,3-thionocarbonate (**11**). The structure proposed for **10** follows from i.r., u.v., and n.m.r. data, and was confirmed by its independent synthesis. Compound **11** had previously[1c] been prepared by treatment of **8** with pyridine. The unsymmetrical dithiobis(thioformate), isolated by preparative t.l.c., was unstable and disproportionated rapidly to give a mixture of **8**, **4**, and **9**. When a portion of this mixture was kept in THF–pyridine for several min, further transformations occurred, to give **10** and **11**.

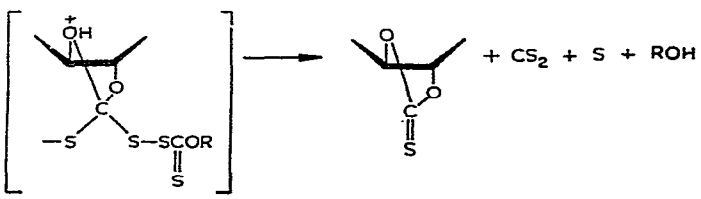
Formation of **10** from **9** could be expected to proceed by the following mechanism.



Displacement by sulfur to the rear of the C–O bond could proceed readily with **9** or with simple dithiobis(thioformates). The latter are known to form *S*-alkyl dithio-

carbonate, carbonyl sulfide, and sulfur[2]. With dithiobis(thioformates) **3** and **8**, displacement by sulfur to the rear of the C–O bond on the furanose or pyranose ring would be difficult, because of steric factors, and it is observed that these derivatives do not decompose to dithiocarbonates.

Formation of **11** could arise from either **8** or **9** *via* a cyclic, orthoester intermediate formed by participation of the C-3 hydroxyl group.



Formation of thionocarbonates by way of an orthoester intermediate has been suggested[1a]. Sugar dithiobis(thioformates) that possess hydroxyl groups adjacent to thioformate groupings, when treated with pyridine, do afford equimolar amounts of thionocarbonate, carbon disulfide, sulfur, and diol[1b].

EXPERIMENTAL

The preparations of bis(1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose) 3,3′-[dithiobis(thioformate)][1a] (**3**) and bis(methyl 4,6-*O*-benzylidene-α-D-glucopyranoside) 2,2′-[dithiobis(thioformate)][1c] (**8**) have been reported earlier. Methyl- and ethylxanthates, prepared by the usual procedures, were crosslinked with iodine to give the corresponding dithiobis(thioformates). Silica Gel G* was used as the absorbent for t.l.c., and sulfuric acid (5%) in methanol, as the spraying agent. For preparative t.l.c., plates were covered with a slurry of 0.5 to 1.0 mm thickness of adsorbent, dried, and then sprayed with Rhodamine B as the indicator. Ethyl acetate–carbon disulfide (1:4, v/v) or acetone–hexane (1:9, v/v) served as the solvent. U.v. spectra were recorded with a Perkin–Elmer Model 202 spectrophotometer. I.r. spectra were recorded for films, with a Perkin–Elmer Model 137 spectrophotometer. Carbonyl sulfide was determined as described previously[3]. N.m.r. spectra were recorded with a Varian HA-100 n.m.r. spectrometer, with pyridine-d_5 as the solvent. Chemical shifts refer to an internal standard of tetramethylsilane ($\tau = 10.00$).

1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose 3-[O-ethyl dithiobis(thioformate)] (**5**). — *A*. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (0.5 g) in methyl sulfoxide (0.5 ml) was treated with carbon disulfide (0.5 ml) and sodium hydroxide (5M, 0.5 ml). The mixture was kept for 10 min at 25°, cooled to 5°, mixed with sodium ethylxanthate (0.5 g), and treated with 0.3M iodine solution. Water (about 50 ml) was added, and

*Mention of a company name or a product by the U. S. Department of Agriculture does not imply approval or recommendation of the company or product to the exclusion of others which may also be suitable.

the mixture was extracted with ether. T.l.c. of the extract revealed three spots: *O,O'*-diethyl dithiobis(thioformate) (revealed under u.v. light on t.l.c. plates sprayed with Rhodamine B), **3**, and a spot having a higher R_F value than **3**. This spot of higher R_F value was extracted with acetone, and identified as **5**.

Anal. Calc. for $C_{16}H_{24}O_7S_4$: C, 42.1; H, 5.3. Found: C, 42.0; H, 5.4.

The u.v. spectrum of **5** showed bands characteristic of dithiobis(thioformate); namely, a broad maximum near 240 nm and a shoulder near 280 nm.

B. A solution of **3** (50 mg) in THF (0.3 ml) was treated with **4** (100 mg), and kept at 25°. Portions were removed periodically for analysis by t.l.c. The ratio of the amounts of **3** and **5** present at various times was determined by removal of the t.l.c. adsorbent from the appropriate zones, followed by elution of the components from the adsorbent with similar volumes of ethanol. The ratio was calculated from the absorption maxima at 240 nm.

*1,2:5,6-Di-*O*-isopropylidene-α-*D*-glucofuranose 3-(S-ethyl dithiocarbonate)* (**6**). — *A.* A solution of **3** (600 mg) in pyridine (0.6 ml) was heated for 1 min to 95°, and then **4** (300 mg) was added dropwise, with stirring, during 5 min. Carbonyl sulfide was evolved, and elemental sulfur was precipitated from the solution. T.l.c. indicated only one sugar component, which was identified as **6**.

Anal. Calc. for $C_{15}H_{24}O_6S_2$: C, 49.5; H, 6.6; S, 17.5. Found: C, 49.2; H, 6.3; S, 17.4.

U.v. spectra showed bands characteristic of *S*-alkyl dithiocarbonate; namely, absorption maxima near 225 and 284 nm.

Alkaline hydrolysis of **6** with barium hydroxide, followed by neutralization with carbon dioxide, gave 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose, m.p. undepressed upon admixture with an authentic sample.

B. 1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose (0.5 g) in methyl sulfoxide (0.5 ml) was treated with carbon disulfide (0.5 ml) and sodium hydroxide (5M, 0.5 ml). The mixture was kept for 10 min at 25°, cooled to 5°, and allowed to stand with iodoethane (5 ml) for 15 min. Water (about 50 ml) was added, and the mixture was extracted with ether. The extract was washed twice with water, dried (Na_2SO_4), and evaporated. The syrup so obtained had the same R_F value and i.r. spectrum as **6**.

Reaction of O,O'*-dimethyl dithiobis(thioformate) with* **3**. — To a solution of **3** (50 mg) in THF (0.2 ml) and pyridine (0.1 ml) was added *O,O'*-dimethyl dithiobis-(thioformate) (100 mg) dropwise. An immediate evolution of carbonyl sulfide and precipitation of elemental sulfur occurred. T.l.c. revealed only one sugar component, which was separated by preparative t.l.c. and identified as 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose 3-(*S*-methyl dithiocarbonate) by sulfur analysis (found 18.2%; theory 18.4%) and by u.v. spectrum (absorption maxima at 225 and 280 nm).

When the experiment was repeated without pyridine, two spots having almost equal intensities appeared on t.l.c. plates. The R_F value of one spot was similar to, and that of the other was greater than, the R_F value of **3**. When the component forming the latter was extracted from preparative t.l.c. plates and the extract was rechromatographed, two spots were found having R_F values similar to those from

the sugar components in the original mixture. The u.v. spectrum of the extract revealed only dithiobis(thioformates).

Reaction of bis(methyl 4,6-O-benzylidene-α-D-glucopyranoside) 2,2'-dithiobis-(thioformate) (**8**) *with* **4**. — A solution of **8** (75 mg) in THF (0.5 ml) was treated with **4** (100 mg) and kept at 25°. Progress of the reaction was followed as described in the formation of **5** part B. The solution was kept for 18 h, and the sugar products were isolated by preparative t.l.c. The major component gave the sulfur analysis expected for the unsymmetrical dithiobis(thioformate) (found, 26.5; theoretical, 26.8). The other sugar component was **8**.

The reaction was repeated, but with THF containing 1% of pyridine. The unsymmetrical dithiobis(thioformate) formed rapidly, and, during 2 h, about 75% of **8** was converted. After the solution had been kept for 18 h, t.l.c. revealed two sugar components, neither of which correspond to **8** or to the unsymmetrical product. Isolation of the components from preparative t.l.c. plates yielded crystalline compounds identified as **10** and **11**. Compound **11** was identified by comparison with an authentic sample[1c]. The identity of **10** was established by comparison with methyl 4,6-*O*-benzylidene-α-D-glucopyranoside 2-(*S*-ethyl dithiocarbonate), prepared as next described.

Preparation of methyl 4,6-O-benzylidene-α-D-glucopyranoside 2-(S-ethyl dithiocarbonate) (**10**). — Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (5 g) in *p*-dioxane (15 ml) was treated with 5M sodium hydroxide (4 ml) and carbon disulfide (10 ml). The mixture was stirred for 15 min, neutralized to pH 8 with 5M acetic acid, and treated with iodoethane (10 ml). After another 15-min interval, the reaction mixture was extracted with ether (50 ml), and the ether extract was dried, and evaporated to a syrup (3.5 g). The syrup was dissolved in ether, and hexane was added to slight turbidity. Upon storing the solution at −10°, crystals were deposited. The first crop was almost pure **10**, and had m.p. 157–158°. Characteristic absorption bands in the i.r. spectrum at 1070 and 1200 cm^{-1} and in the u.v. spectrum at 228 nm (ε 6,500) and 288 nm (ε 10,100) identified the dithiocarbonate ester structure. N.m.r. data: τ 3.92 (1-proton quartet, $J_{2,3}$ 5.0 Hz, H-2), τ 4.25 (1-proton singlet, benzylidene-H), τ 4.61 (1-proton doublet, $J_{1,2}$ 1.9 Hz, H-1), τ 5.25 (1-proton triplet, $J_{3,4}$ 5.0 Hz, H-3), τ 5.62 (1-proton quartet, H-5), τ 5.89 (1-proton triplet, $J_{4,5}$ 5.0 Hz, H-4), τ 6.05–6.25 (2-proton multiplet, H-6,6'), τ 6.69 (3-proton singlet, OMe), and τ 6.95 and 8.80 (2-proton quartet and 3-proton triplet, respectively, OEt).

Subsequent crops of crystals were mixtures of **10** with the 3-isomer.

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Note

An improved procedure for oxidation of carbohydrate derivatives with ruthenium tetraoxide

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The biological importance of ketoses and their utility in the synthesis of branched-chain and amino sugars has led in recent years to several studies of procedures for oxidation of partially substituted carbohydrates. Work in this laboratory[1] has centered on the ruthenium tetraoxide oxidation method, which was introduced to carbohydrate chemistry by Overend and co-workers[2]. The method has been applied to carbohydrates *O*-substituted with methoxycarbonyl, benzoyl, benzylidene, isopropylidene, and methylene groups, and to alkyl glycosides and 1,6-anhydro sugars[1-6]. The procedure reported by Overend and co-workers[2,6] involves two steps, generation of the oxidant with sodium periodate and ruthenium dioxide, and oxidation of the alcohol with the tetraoxide. Prolonged treatment of some aldofuranose derivatives with an excess of ruthenium tetraoxide has been reported[3] to yield lactones by an oxygen-insertion reaction of the glycosuloses formed initially. In our original procedure[1], the two steps are combined; a solution of the carbohydrate in chloroform or carbon tetrachloride is treated with a catalytic amount of ruthenium dioxide (20 mg/g of sugar), and a 5% aqueous solution of sodium periodate (1.3 equivalents) is added with vigorous stirring and control to pH 6–7 (sodium hydrogen carbonate). As a result of requests from several workers, we now report a modification of this procedure in connection with some oxidation studies that demonstrate the stability of additional functional groups (see Table I) towards ruthenium tetraoxide.

In the improved procedure, the problem of over-oxidation is minimized by use of the sparingly soluble potassium periodate, and the pH is controlled by the addition in bulk of a determined quantity of potassium carbonate. Ruthenium dioxide is formed from ruthenium trichloride (British Drug Houses Ltd.) by the precipitation method described by Müller and Schwabe[7]. For oxidation of "isolated" secondary hydroxyl groups to ketones in protected sugar derivatives, the compound is dissolved in ethanol-free chloroform to give an approximately 15% solution, and an equal volume of water is added. For each mole of substrate, 0.24 mole of anhydrous potassium carbonate, 1.3 moles of potassium periodate, and a catalytic amount (~0.05 mole) of ruthenium dioxide are then added. The reaction mixture is stirred vigorously, and the progress of the oxidation is followed by t.l.c.*. At the end of the reaction, the mixture is treated

TABLE I

OXIDATION OF CARBOHYDRATE DERIVATIVES WITH RUTHENIUM TETRAOXIDE

Substrate	*Product*	*Yield, %*
1,2:5,6-Di-*O*-isopropylidene-α-D-glucofuranose	1,2:5,6-di-*O*-isopropylidene-α-D-*ribo*-hexos-3-ulose[2]	90–95
Methyl 4,6-*O*-benzylidene-2-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside[8]	methyl 4,6-*O*-benzylidene-2-*O*-*p*-tolylsulfonyl-α-D-*ribo*-hexopyranosid-3-ulose[9–11]	85
Methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*lyxo*-hexopyranoside[5,12]	methyl 4,6-*O*-benzylidene-2-deoxy-α-D-*threo*-hexopyranosid-3-ulose[2,5]	89
Methyl 2,3-di-*O*-methyl-6-*O*-*p*-tolylsulfonyl-α-D-glucopyranoside[13]	methyl 2,3-di-*O*-methyl-6-*O*-*p*-tolylsulfonyl-α-D-*xylo*-hexopyranosid-4-ulose[a,b]	83
Methyl 2,3-di-*O*-benzoyl-β-L-arabinopyranoside[14,15]	methyl 2,3-di-*O*-benzoyl-β-L-*threo*-pentopyranosid-4-ulose[b,c]	93

[a]Syrupy product, characterized as the crystalline (2,4-dinitrophenyl)hydrazone. [b]To be published; see ref. 16. [c]Characterized by conversion into methyl α-D-xylopyranoside.

with isopropyl alcohol to reduce residual ruthenium tetraoxide. The mixture is filtered, and the aqueous layer is extracted with chloroform. The combined extracts are dried, and concentrated to give the oxidized product. The oxidations listed in Table I have been achieved with this improved procedure.

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*If oxidation is incomplete, further amounts of potassium carbonate, potassium periodate, and ruthenium dioxide may be added in the specified ratio.

Note

The direct preparation of 1-thio-D-glucitol and its disulfide from D-glucose

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Novel, alkaline pulping-processes that have been developed in these laboratories involve the treatment of wood chips with hydrogen sulfide at elevated temperatures and pressures, prior to alkaline pulping[1]. The nature of the wood-carbohydrate reactions on treatment with hydrogen sulfide is at present under investigation, and, in this report, the results of some of our initial studies with D-glucose as a model compound are described.

The formation of thio sugars is to be expected in the reaction of D-glucose with hydrogen sulfide under pressure[2-5]. Specifically, the formation of 1-thio-D-glucose has been reported when 1,2-anhydro-α-D-glucopyranose is treated with hydrogen sulfide[6]. The reaction of carbonyl compounds with hydrogen sulfide is fairly complex, and it has been reported that hydroxy thiols, *gem*-dithiols, and vinyl thiols, can be formed[7]. More recently, the equilibrium kinetics and mechanism of the addition of thiols to the carbonyl group have also been studied[8].

Treatment of an aqueous solution of D-glucose with hydrogen sulfide at elevated temperatures and pressures resulted in the formation of 1-thio-D-glucitol (**1**) and its disulfide (**2**) as major products. An authentic sample of **1** was prepared by treating an aqueous solution of D-glucose with hydrogen at 1000 $lb.in^{-2}$ in the presence of cobalt, polysulfide normally employed as a catalyst[4]. Subsequent oxidation of **1** with iodine yielded an authentic sample of **2**. Compounds **1** and **2**, isolated from the hydrogen sulfide treatment of D-glucose, were identified by paper-chromatographic mobility and by the m.p. and mixed m.p. data of certain derivatives.

This novel method of preparing 1-thioalditols is much simpler than methods[4] previously known, and affords a ready route, by bromine oxidation, to the corresponding oxysulfur compounds[9], and to deoxy carbohydrates by reductive desulfurization with Raney nickel[10]. A similar reductive thiolation at the reducing end-group takes place in the presence of sodium carbonate, and has been utilized[11] to stabilize cellulose to alkaline degradation.

In view of this reaction, it would seem likely that the mechanism of the cobalt polysulfide-catalyzed hydrogenation of D-glucose involves the production of hydrogen sulfide, as suggested by Farlow *et al.*[4]. It was established that **2** is reduced to **1** under the hydrogen sulfide reaction-conditions and, stoichiometrically, the overall reaction

may be represented as:

$$\underset{\text{(D-Glucose)}}{2\,C_6H_{12}O_6} + 3\,H_2S \rightarrow \underset{\textbf{(2)}}{C_{12}H_{26}O_{10}S_2} + 2\,H_2O + S$$

$$\underset{\textbf{(2)}}{C_{12}H_{26}O_{10}S_2} + H_2S \rightarrow 2\,\underset{\textbf{(1)}}{C_6H_{14}O_5S} + S$$

The simultaneous direct formation of (1) by the reductive thiolation of D-glucose is also likely[11].

$$\underset{\text{(D-Glucose)}}{C_6H_{12}O_6} + 2\,H_2S \rightarrow \underset{\textbf{(1)}}{C_6H_{14}O_5S} + H_2O + S$$

EXPERIMENTAL

General methods. — Melting points were determined with a Fisher–Johns apparatus, and are corrected. U.v. spectra were recorded with a Unicam model SP 800. I.r. spectra were recorded with a Perkin–Elmer Model 521 spectrophotometer. Paper chromatograms were performed by downward irrigation on Whatman No. 54 paper, with 4:18:3:1 (v/v) water–ethyl acetate–acetic acid–formic acid as eluant. Visibilization was achieved by spraying in succession with 0.05% aqueous sodium metaperiodate and a solution of benzidine dihydrochloride[12]. The mobilities of carbohydrates are expressed as R_G values, relative to D-glucose as 1.00.

1-Thio-D-glucitol (1). — In a 1000-ml, Parr pressure-reaction vessel (Parr Instrument Company, Moline, Illinois) was placed a solution of D-glucose (60 g) in water (330 ml), and the vessel was purged free of air with nitrogen. Hydrogen sulfide was passed in, to a pressure of about 70 lb.in^{-2}, and the vessel was heated to 150°. As hydrogen sulfide was consumed, it was continuously replenished to a pressure of 170 lb.in^{-2}. The reaction was followed by paper chromatography of samples of the reaction mixture, withdrawn through a sampling valve. The gradual build-up of products having R_G values of 2.4 and 0.61, corresponding to **1** and its disulfide (**2**), was observed. The samples were also analyzed quantitatively for thiol, by titration with standard iodine after removal of hydrogen sulfide by aspiration. The total amount of thiol and disulfide was determined by a similar titration after reduction of sample aliquots with zinc dust. This quantitative analysis confirmed the simultaneous formation of **1** and **2** in the reaction.

After 150 min at the reaction temperature and pressure, the vessel was cooled, and the gas was vented into a trap containing aqueous sodium hydroxide. The total product was isolated as the thiol by first boiling the reaction solution with zinc dust for 15 min to reduce the disulfide to the zinc salt of the thiol. The mixture was filtered free from zinc, the filtrate was concentrated, and the zinc salt was precipitated by adding 10 volumes of methanol. The white product was filtered off, washed with methanol, and suspended in 90% aqueous ethanol. The free thiol was regenerated by treating this suspension for 10 min with hydrogen sulfide at 100 lb.in^{-2} and 40°. The zinc sulfide was filtered off, and the product was obtained by evaporating

the filtrate to a syrup under diminished pressure. The yield, as determined by iodine titration for the thiol product, was 30 g (50%). Solid **1** was obtained as white, hygroscopic material by crystallization from cold, absolute ethanol.

S-*Dodecyl-1-thio*-D-*glucitol* (**3**). — Compound **1** (1 g) was dissolved in water (2.5 ml) and sodium hydroxide (0.2 g) was added. To this mixture was added 1-bromododecane (1.25 g) in *p*-dioxane (2.5 ml), and the resulting mixture was refluxed for 2 h. Solid **3** separated on cooling, and was successively washed with water, *p*-dioxane, and ether; yield 1.5 g (80%), m.p. 106°, unchanged on admixture with authentic[4] **3**.

1,1'-Dithiobis(1-*deoxy*-D-*glucitol*) (**2**). — The thiol **1** (5.0 g) was dissolved in hot ethanol (25 ml), and treated with an equivalent amount of an alcoholic solution of iodine. Pure **2** was obtained as colorless needles by crystallization from ethanol; yield 4.5 g (90%), m.p. 129°, unchanged on admixture with authentic[4] **2**; $\lambda_{max}^{H_2O}$ 247 nm (ε 354). Similar u.v. spectral properties have been found for other aliphatic disulfides[13].

The disulfide **2** was also obtained in good yield by direct crystallization from the reaction solution of some preparations, after changing the solvent to methanol.

1,1'-Dithiobis(*1-deoxy*-D-*glucitol*) *decaacetate* (**4**). — A mixture of **2** (2.0 g) and fused sodium acetate (1.0 g) was dissolved in 10 ml of acetic anhydride, and refluxed for 3 h. The cooled mixture was added to 20 ml of ice–water, and the crude product was filtered off. Pure **4** was obtained as colorless needles by recrystallization from methanol; yield 3.0 g (75%), m.p. 120–121°, unchanged on admixture with authentic[4] **4**; $\lambda_{max}^{CHCl_3}$ 5.73 μm (OAc); the SAc band[14] at 5.89 μm was absent.

Authentic 2,3,4,5,6,-penta-*O*-acetyl-1-*S*-acetyl-1-thio-D-glucitol (**5**) was prepared from the sample of **1** that had been prepared by the catalytic hydrogenation method[4], m.p. 87°; $\lambda_{max}^{CHCl_3}$ 5.73 (OAc) and 5.89 μm (SAc)[14]. Attempts to prepare **5** from samples of **1** prepared by the hydrogen sulfide method were not always successful; this failure was attributed to the hygroscopic nature of the starting material.

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Note

An improved method of synthesis of *o*-nitrophenyl β-D-galactopyranoside 6-phosphate*

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In a previous communication[1] we described the synthesis of *o*-nitrophenyl β-D-galactoside 6-phosphate (**1**), a substrate for the staphylococcal β-D-galactoside 6-phosphate galactohydrolase (phospho-β-galactosidase). The rather complicated reaction-sequence used involved the synthesis of a partially protected derivative of *o*-nitrophenyl β-D-galactoside (**2**) followed by the removal of different types of protecting groups, such as trimethylsilyl and β-cyanoethyl.

The staphylococcal 6-phospho-β-D-galactosidase has been shown to be a useful analytical tool for the determination of the components of the staphylococcal phosphoenol pyruvate-dependent phosphotransferase system[2]. This system plays a major role in the accumulation and metabolism of many carbohydrates, including galactosides in *Staphylococcus aureus*[3]. In addition, the 6-phospho-β-D-galactosidase has been found only in *Staphylococcus* and some closely related microorganisms.

A procedure for phosphorylating primary hydroxyl groups in unprotected nucleosides has been described[4]. Phosphorus oxychloride in trimethyl phosphate containing small proportions of water converted nucleosides into the 5′-phosphates at 0°, while the 2′ and 3′-phosphates were observed only in trace amounts. We applied the method of phosphorylation described upon the unprotected glycoside **2** and obtained the desired 6-phosphate **1** in 50% yield. Some glycoside **2** was degraded during the reaction. The neutralized reaction mixture contained large amounts of inorganic phosphate and chloride, which were removed by absorption of **1** and the unconverted glycoside **2** on a charcoal–Celite mixture, with subsequent elution of the salts with water. The phosphate **1** and traces of **2** were then eluted with a pyridine–water mixture. The dicyclohexyl ammonium salt of **1** crystallized readily from ethanol.

The position of the phosphate ester at the primary hydroxyl group on the

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*This investigation was supported by research grant GB-5520 from the National Science Foundation Washington, D. C., and Life Insurance Medical Research Fund G-66-32.

D-galactose moiety of **1** was confirmed by the fact that (*a*) the rotational data agreed with the product synthesized earlier[1]; and (*b*) D-galactose 6-phosphate obtained by the cleavage of **1** with purified 6-phospho-galactosidase showed electrophoretic properties identical with those of commercial D-galactose 6-phosphate in a borate buffer system.

EXPERIMENTAL

o-Nitrophenyl-β-D-galactoside 6-phosphate (**1**). — 30 mmoles of *o*-nitrophenyl β-D-galactoside[5] (**2**) was dissolved in 75 ml of trimethyl phosphate (Aldrich Chemical Co.) containing 30 mmoles of water and 90 mmoles of phosphorus oxychloride. The mixture was stirred and kept for 3 h in an ice bath. Crushed ice was then added, phosphoric and hydrochloric acids were neutralized with concentrated ammonia, and the yellow solution was evaporated under diminished pressure. *o*-Nitrophenol was removed by evaporating water several times from the solution. The colorless solution contained 14.65 mmoles of **1** and 3.7 mmoles of **2** as determined enzymically. Alkaline hydrolysis of the reaction mixture gave 18.2 mmoles of product indicating no substantial formation of by-products, since **1**+**2** = 18.35 mmoles. Compounds **1** and **2** were absorbed on an acid-washed, 1:1 charcoal–Celite mixture. The supernatant of the charcoal absorption was checked enzymically for residual phosphate **1**. To absorb **1** and **2**, ~1000 ml of wet charcoal–Celite mixture were needed. After absorption of the glycosides on the charcoal, the slurry was poured into a column of 10 cm diameter. The inorganic salts were eluted with 4–5 liters of water until no chloride ion was detectable. With about 3 liters of 1:2 (v/v) pyridine–water, the phosphate **1**, containing traces of **2**, was eluted with a small loss of **1**. After evaporation the mixture was passed through a column of Dowex-50 (H^+ form). The eluate was brought to pH 9 with cyclohexylamine, evaporated to a syrup and dissolved in a small volume of ethanol. Addition of ether until the solution became cloudy produced crystals of the dicyclohexylammonium salt; yield 13 mmoles. The product decomposed at ~180°, and had $[\alpha]_D^{20}$ −40° (*c* 2, water); Calc. for the free acid, $[\alpha]_D^{20}$ −60.6°.

Anal. Calc. for $C_{24}H_{43}N_3O_{11}P$ (dicyclohexylammonium salt): C, 49.65; H, 7.47; N, 7.24; P, 5.33. Found: C, 49.31; H, 7.42; N, 7.25; P, 5.63. Enzymic analysis with 6-phospho-β-D-galactosidase, found: 4.06×10^{-6}M. Calc: 4.18×10^{-6}M.

Analytical procedures. — Most of the procedures used were described previously[1]. Enzymic determination of the phosphate **1** was performed with 10 μg of a highly purified, electrophoretically homogeneous, enzyme preparation in 2.5 ml of the buffer described earlier[1]. For high-voltage paper electrophoresis, 3 mg of **1**, as the dicyclohexylammonium salt, was hydrolyzed in the standard buffer with 10 μg of 6-phosphogalactosidase. The solution was applied to a Whatman 3 MM paper strip of 120 cm in length and subjected to electrophoresis for 1 h at 33 V/cm in an 0.05M sodium borate buffer[6], pH 9.5, at 30°. The apparatus used was a Gilson High-Voltage Electrophorator, Model DW. The spots on the paper were made visible by spraying with alkaline silver nitrate solution.

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Preliminary communication

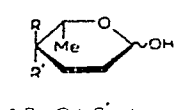
STUDIES INTO THE SYNTHESIS OF TRIDEOXY SUGARS

A.H. HAINES

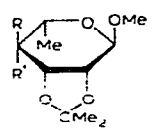
School of Chemical Sciences, University of East Anglia, University Plain, Norwich, NOR 85C (Great Britain)

(Received March 31st, 1969)

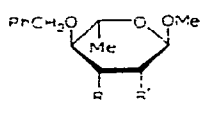
The antibiotics amicetin[1], rhodomycin[2], and streptolydigin[3] contain 2,3,6-trideoxyaldohexoses. Stevens *et al.*[4] have synthesised 2,3,6-trideoxy-D-*erythro*-aldohexose (amicetose, the enantiomer of compound **1**), which occurs in amicetin, from tri-*O*-acetyl-D-glucal; the formation of the 6-deoxy group was the penultimate step. In this communication, a new approach to the synthesis of these types of trideoxy sugar and their derivatives is reported, which commences with a 6-deoxy sugar, and utilises the Corey—Winter olefin synthesis proceeding from *cis*-vicinal diols[5].



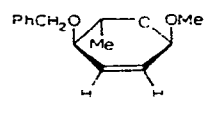
1 R = OH, R' = H
2 R = H, R' = OH



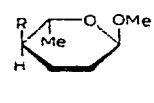
3 R = OH, R' = H
4 R = OCH_2Ph, R' = H
5 R = H, R' = OH



6 R = R' = OH
7 R, R' = O, O, C=S



8



9 R = OCH_2Ph
10 R = OH
11 R = $OCO{\cdot}C_6H_3(NO_2)_2$

Methyl 4-*O*-benzyl-6-deoxy-2,3-*O*-isopropylidene-α-L-mannopyranoside (**4**), b.p. 146—147°/0.8 mm, $[\alpha]_D$ -62.6° (*c* 3.8, acetone), was obtained by benzylation of the corresponding alcohol[6] **3**. Partial hydrolysis of the ether **4** with 0.05N hydrochloric acid at 100° yielded methyl 4-*O*-benzyl-6-deoxy-α-L-mannopyranoside (**6**), m.p. 107—109°, $[\alpha]_D$ -68.3° (*c* 1.6, chloroform). Reaction of the diol **6** with bis(imidazol-1-yl)thione[5] yielded the syrupy thionocarbonate **7**, $[\alpha]_D$ -50.2° (*c* 2.1, chloroform), which was treated immediately with trimethylphosphite[5] to yield methyl 4-*O*-benzyl-6-deoxy-α-L-*erythro*-hexopyran-2-enoside (**8**), b.p. 124—128°/0.8 mm, $[\alpha]_D$ -168° (*c* 2.1, chloroform). Platinum-catalysed reduction of the olefin **8** yielded methyl 4-*O*-benzyl-2,3,6-trideoxy-α-L-*erythro*-hexopyranoside (**9**), b.p. 114—115°/0.5 mm, $[\alpha]_D$ -175° (*c* 2.2, chloroform). Hydrogenolysis of the benzyl group, catalysed by palladium on charcoal, gave methyl 2,3,6-trideoxy-α-L-*erythro*-hexopyranoside (**10**), b.p. 97—99°/12 mm, $[\alpha]_D$ -144° (*c* 0.23, chloroform), which is the methyl glycoside of the enantiomer of natural amicetose. The glycoside afforded a 3,5-dinitrobenzoate (**11**), m.p. 96—98°, $[\alpha]_D$ -119° (*c* 0.9, chloroform).

T.l.c. studies showed that the glycoside **10** was readily hydrolysed to the free sugar. The 2,4-dinitrophenylhydrazone of this sugar was prepared from the glycoside in 84% yield; m.p. 154–156°, $[\alpha]_{546}$ +11.3° (*c* 0.23, pyridine). The 2,4-dinitrophenylhydrazone[4] of the natural sugar has m.p. 156–157°, $[\alpha]_D$ -10.0° (*c* 0.9, pyridine).

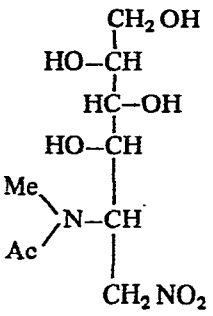
An extension of this study was made to the synthesis of 2,3,6-trideoxy-L-*threo*-aldohexose (**2**) (natural rhodinose), which occurs in rhodomycin and streptolydigin; Stevens *et al.*[4] have reported the preparation of the enantiomer of natural rhodinose. A similar sequence of reaction was carried out on methyl 6-deoxy-2,3-*O*-isopropylidene-α-L-talopyranoside (**5**), which was obtained by reduction (lithium aluminium hydride) of methyl 2,3-*O*-isopropylidene-α-L-*lyxo*-hexopyranosid-4-ulose[7]. The details of this synthesis will be reserved for the full paper. All new compounds gave satisfactory elemental analyses.

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CORRIGENDA

Carbohydrate Research, 9 (1969) Structure (2) on page 125 should read:

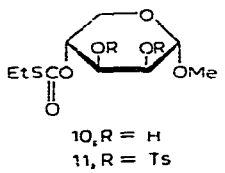


Carbohydrate Research, 10 (1969)

Page 227, line 15–17 should read:

τ 6.91 (3-proton singlet, CH_3SO_3-), τ 6.90 (multiplet, 2 protons, H-6a,6b), τ 6.10 (1-proton broad doublet, $J_{3,4}$ 2.7 Hz, H-3), τ 5.56 (1-proton doublet, $J_{1,2}$ 2.5 Hz), τ 5.14 (multiplet, 2 protons, H-4, H-5), τ 4.06 (1 proton, H-1).

Page 290, in the second row of formulae, 10, 11 should be:



ADDENDUM

Carbohydrate Research, 6 (1968) Page 383:

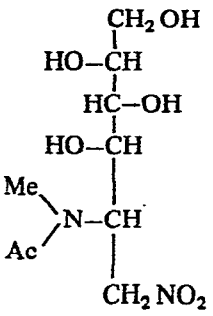
Concerning the conversion of α-D-mannopyranose into β-D-mannopyranose, the addition of 7 ml of acetic acid instead of 2 ml has induced a more rapid crystallisation. This latter procedure has produced 6.5 g of β-D-mannopyranose when the solution was nucleated, and the vessel scratched with a glass rod, and kept overnight at room temperature and for 24 h at 4°.

ANNOUNCEMENT

The International Symposium on Blood and Tissue Antigens will be held in Ann Arbor, Michigan (U.S.A.) on September 17–19, 1969. For information, write to Dr. D. Aminoff, The Thomas Henry Simpson Memorial Institute for Medical Research, The University of Michigan, Ann Arbor, Mich. 48104 (U.S.A.).

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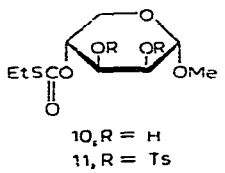


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O R D CHARACTERISTICS OF AZIDO DERIVATIVES OF PENTOSES

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(Received September 26th, 1967, in revised form, December 30th, 1968)

ABSTRACT

O r d curves of some methyl 4-azido-4-deoxypentopyranosides have been measured Although it is not possible to predict, on the basis of analysis by an azide octant rule, the sign of the Cotton effect exhibited by these derivatives, several empirical observations are noteworthy Those derivatives in the *C1* (D) conformation and having an axial azide substituent exhibit positive Cotton effects, whereas those having an equatorial azide substituent show negative Cotton effects Azide derivatives in the *1C* (D) conformation and having an equatorial azide group exhibit a negative Cotton effect, whereas those having an axial azide substituent show a positive Cotton effect There is a marked dependence of the magnitude of the background rotation on the configuration of the substituent at C-1, an axial, glycosidic, methoxyl group causes a much higher background rotation than does an equatorial substituent

INTRODUCTION

Carbohydrate azides are readily available by nucleophilic substitution reactions of the appropriate sulphonates by azide ion[1-7], and by ring opening of suitable epoxides and epimines[8,9]. Their synthetic utility lies in the fact that they are easily hydrogenated to amino sugars

The inherently symmetric[10] azide chromophore, which, in alkyl azides, exhibits a weak transition[11] (log ε 1 3) at 280–290 nm, should be useful for correlating optical activity with molecular structure Ripperger[12] and Djerassi *et al* [13] have described an "octant rule" for the transition of the azide chromophore at 280–290 nm The latter group[13] have attempted to apply the rule to predict the sign of the Cotton effect of steroid azides, and have drawn an analogy with the octant rule applied to the carbonyl group of saturated ketones[15] for specification of the sign to be associated with a particular octant In essence, the chromophore is viewed along the N–N–N axis from N-3 toward N-1, with the bond specifying the lone pair of electrons lying in a vertical plane From this viewpoint, the signs of the azide octants are the same as those of the carbonyl octants

EXPERIMENTAL

U v and o r d curves (for 0 1% solutions in ethanol at 25–26°) were measured

on a JASCO ORD/UV-5 spectropolarimeter, with an accuracy of ±1 nm in the range 400–235 nm. The cell path-length was 1–10 mm Most of the compounds employed have been described elsewhere (see Table I)

RESULTS AND DISCUSSION

A number of 4-azido-4-deoxy derivatives of methyl pentopyranosides possessing the *xylo* and *arabino* configuration were available from a study of synthetic routes leading to 4-acetamido-4-deoxypentoses[5,6 14] The introduction of the azide function was accomplished by treatment of derivatives of methyl 4-*O*-methanesulphonylpentopyranosides with sodium azide in *N,N*-dimethylformamide The compounds for which o r d curves were measured are listed in Table I, and the o r d data are included in Table II It is assumed that, irrespective of whether a compound contains free or substituted hydroxyl groups, it exists in the same preferred conformation (Table III) This assumption is justified because of the similar shape of the o r d curves of the pairs of compounds: **3–4**, **6–7**, **8–9**, and **13–14** (Figs 1–3)

The conformation of the cyclohexane ring is the only stereochemical factor taken into consideration when cyclohexanones are investigated by means of the octant rule; the situation is much more complicated for azides because of the free-rotating chromophore At this stage, it is therefore not possible to predict the sign of the Cotton effect of these azides on the basis of the octant rule

The rotation strength of the azide chromophore is much weaker than that of the carbonyl group[13]. For this reason, the Cotton effects in the o r.d. curves of

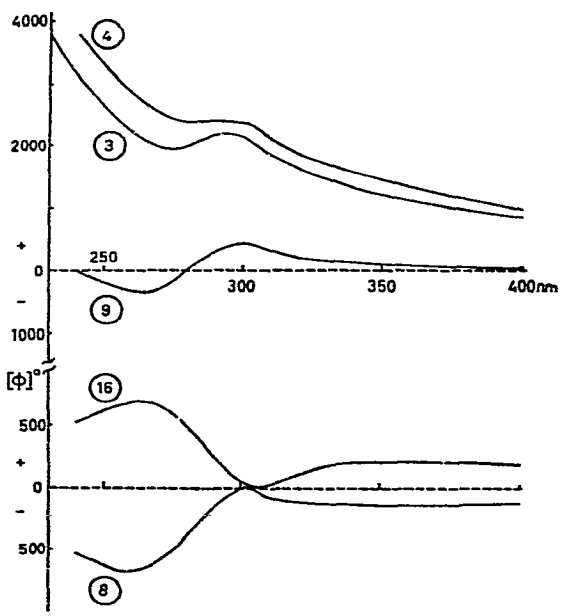


Fig 1 Anomeric and enantiomeric derivatives of methyl 4-azido-4-deoxyarabinopyranoside.

many of the azides are discernible only with difficulty, especially in those having a high background rotation[16]

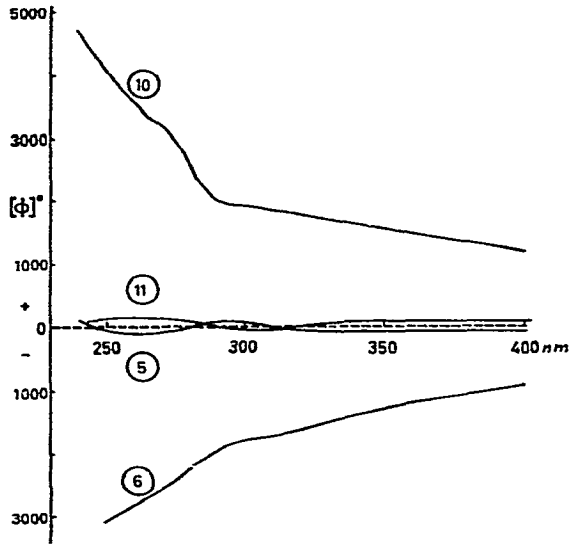


Fig 2 Anomeric derivatives of methyl 4-azido-4-deoxyxylopyranoside

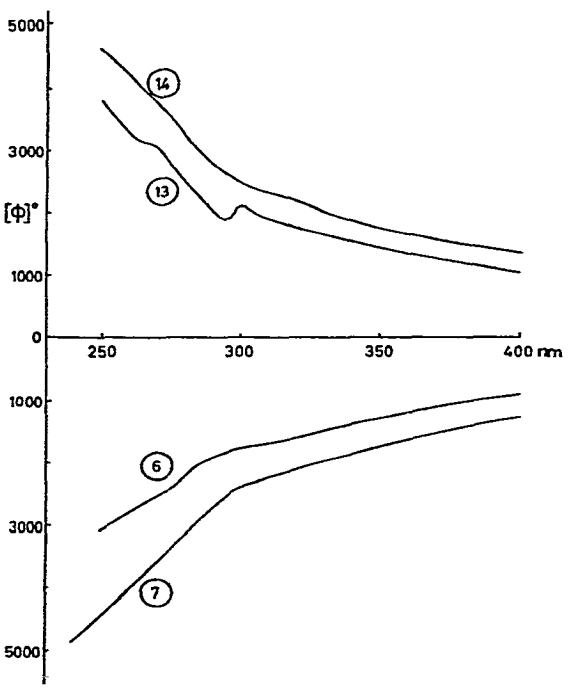


Fig 3 Enantiomeric derivatives of methyl 4-azido-4-deoxyxylopyranoside

TABLE I

THE AZIDO DERIVATIVES OF METHYL PENTOPYRANOSIDES

No.	*Methyl 4-azido-4-deoxypyranoside*	*Preferred conformation*	*Orientation of azide group*	*Sign of Cotton effect*
1	3-*O*-Acetyl-2-*O*-mesyl-β-L-*arabino*-[17]	*C1*	axial	+
2	2,3-Anhydro-β-L-*ribo*-[5]			
3	2,3-Di-*O*-mesyl-β-L-*arabino*-[5]	*C1*	axial	+
4	β-L-*arabino*-[5]	*C1*	axial	+
5	β-L-*xylo*-[5]	*1C*	equatorial	+
6	α-L-*xylo*-[14]	*1C*	equatorial	+
7	3-*O*-Methyl-α-L-*xylo*-[14]	*1C*	equatorial	+
8	α-L-*arabino*-[14]	*C1*	axial	+
9	2,3-Di-*O*-mesyl-α-L-*arabino*-[6]	*C1*	axial	+
10	2,3-Di-*O*-mesyl-α-D-*xylo*-[6]	*C1*	equatorial	−
11	2,3-Di-*O*-mesyl-β-D-*xylo*-[6]	*C1*	equatorial	−
12	2,3-Anhydro-α-D-*ribo*-[6]			
13	α-D-*xylo*-[6]	*C1*	equatorial	−
14	3-*O*-Methyl-α-D-*xylo*-[14]	*C1*	equatorial	−
15	2,3-Di-*O*-benzoyl-α-D-*xylo*-[17]			
16	α-D-*arabino*-[14]	*1C*	axial	−

All of the compounds (**1**, **3**, **4**, **6**, **7**, **10**, **13**, and **14**, Table III) containing an axial methoxyl group at C-1 have a high background rotation which parallels their high specific rotation at the sodium D line On the other hand, derivatives with an equatorial methoxyl group at C-1 show well-defined Cotton effects Thus, compounds (*e g* , **8**) in the *C1* (D) conformation with an axial azide substituent show positive Cotton effects, and those (*e g* , **11**) with an equatorial azide group show negative Cotton effects The situation in the *1C* (D) conformation is reversed Thus, compound **16** (axial azide) shows a negative Cotton effect, and compound **5** (equatorial azide) shows a positive Cotton effect The corresponding anomers of these compounds (with an axial C-1 methoxyl group), which have a high background rotation, show Cotton effects of the same sign, because they possess the same conformation and orientation of the azide group (Table I)

The sign of the Cotton effect is not influenced by substitution of the hydroxyl groups, but some background changes are noticeable

The anomalous character of compound **15** cannot be distinguished in the spectrum, because of overlapping involving the strong absorption of the benzoyl groups

Compounds **2** and **12** are anhydro derivatives, and hence a correlation with the findings mentioned above cannot be made

A comparison of the o r d curves of anomers, enantiomers, and epimers deserves mention Of the anomeric L-arabinose derivatives, the β-L anomers **3** and **4** (Fig 1) have significantly higher positive values of optical rotation in the whole range measured than do the corresponding α-L anomers **8** and **9** A similar situation applies for the anomeric L-xylose derivatives **5** and **6** (Fig 2)

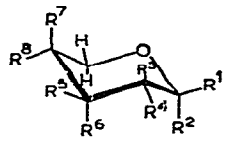
TABLE II

O R D AND U V DATA FOR ETHANOLIC SOLUTIONS OF AZIDO DERIVATIVES OF PENTOPYRANOSIDES

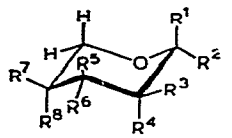
Compound	[∅]	*O r.d* (λ, *nm*)	*U.v* λ_{max}	log ε_{max}	*Compound*	[∅]	*O r d* (λ, *nm*)	*U.v* λ_{max}	log ε_{max}
1	+2600 pk[a]	292	282	1 40	**8**	+ 20 pk	302	284	1.49
	+2460 tr	278				− 690 tr	259		
	+3990 l	220				− 530 l	240		
2	− 490 tr	302	284	1 63	**9**	+ 260 pk	300	283	1 45
	− 100 pk	272				− 320 tr	265		
	− 850 l	240				0 l	240		
3	+2210 pk	294	282	1 49	**10**	+1990 sh	292	283	1 54
	+1970 tr	275				+3110 sh	272		
	+3800 l	230				+4660 l	240		
4	+2400 pk	292	282	1 53	**11**	+ 190 sh	300	283	1.45
	+2390 tr	280				+ 640 sh	273		
	+3800 l	240				+ 930 l	240		
5	+ 90 pk	293	283	1 51	**12**	+2400 sh	302	284	1.45
	− 110 tr	260				+2810 sh	279		
	+ 90 l	240				+5130 l	240		
6	−1730 sh	304	285	1 32	**13**	+1890 sh	296	283	1 44
	−2380 sh	276				+3030 sh	271		
	−3060 l	250				+3780 l	250		
7	−2320 sh	306	285	1 40	**14**	+2440 sh	302	284	1 44
	−3420 sh	274				+3710 sh	272		
	−4880 l	240				+4620 l	250		
					16	+ 10 tr	305	285	1 36
						+ 690 pk	259		
						+ 530 l	240		

[a]Abbreviations pk, peak; tr, trough; sh, shoulder, l, lowest wavelength measured

TABLE III



Compound	R1	R2	R3	R4	R5	R6	R7	R8
1	H	OCH_3	H	OMs	OAc	H	N_3	H
3	H	OCH_3	H	OMs	OMs	H	N_3	H
4	H	OCH_3	H	OH	OH	H	N_3	H
8	OCH_3	H	H	OH	OH	H	N_3	H
9	OCH_3	H	H	OMs	OMs	H	N_3	H
10	H	OCH_3	H	OMs	OMs	H	H	N_3
11	OCH_3	H	H	OMs	OMs	H	H	N_3
13	H	OCH_3	H	OH	OH	H	H	N_3
14	H	OCH_3	H	OH	OCH_3	H	H	N_3



Compound	R1	R2	R3	R4	R5	R6	R7	R8
5	H	OCH_3	OH	H	H	OH	N_3	H
6	OCH_3	H	OH	H	H	OH	N_3	H
7	OCH_3	H	OH	H	H	OCH_3	N_3	H
16	H	OCH_3	OH	H	H	OH	H	N_3

Enantiomeric compounds, as expected, show reflected o.r d curves. For example, in the *arabino* series, the o r d curve of compound **16** (α-D configuration, *C1* conformation) is the mirror image of that of compound **8** (α-L configuration, *1C* conformation), as shown in Fig 1 An analogous situation obtains (Fig 3) for the antipodal *xylo* derivatives **13–6** and **14–7**

Compounds (*e g*, **10–3**, **13–4**, **11–9**, and **16–5**) which are epimeric at C-4 show Cotton effects of the opposite sign

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TOLUENE-*p*-SULPHONYLATION OF METHYL 6-*O*-TRITYL-α-D-MANNOPYRANOSIDE: SYNTHESIS OF 2,4-DI-*O*-METHYL-D-MANNOSE*

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ABSTRACT

Toluene-*p*-sulphonylation of methyl 6-*O*-trityl-α-D-mannopyranoside produced mainly the 3-*O*-toluene-*p*-sulphonyl derivative. Successive methylation, desulphonylation, detritylation, and hydrolysis of this compound yielded 2,4-di-*O*-methyl-D-mannose. A proof of structure of this methylated sugar is presented.

INTRODUCTION

In a previous communication[1], synthetic 3,5-di-*O*-methyl-D-mannose was shown not to be identical with the compound previously assigned this structure[2]. It was suggested by a Referee that the compound previously reported[2] as 3,5-di-*O*-methyl-D-mannose could be 2,4-di-*O*-methyl-D-mannose, and a synthesis of this sugar was therefore undertaken We now report that the di-*O*-methyl-D-mannose reported by Urbas *et al* [2], and subsequently by others[3-6], was in fact 2,4-di-*O*-methyl-D-mannose

RESULTS AND DISCUSSION

Selective sulphonylation of methyl 6-*O*-trityl-α-D-mannopyranoside yielded mainly the 3-*O*-tosyl† compound (overall yield, 51%), with only a small proportion of a mixture of two monosulphonates (17%) and a disulphonate (2%) (*cf* the behaviour[1] of methyl 6-*O*-trityl-α-D-mannofuranoside). The major sulphonate was successively methylated, detosylated, detritylated, and hydrolysed to yield a crystalline dimethyl ether, which, on demethylation, gave mannose. The di-*O*-methyl-D-mannose was found to be identical (paper chromatography, paper electrophoresis, colour reaction with aniline hydrogen phthalate, $[\alpha]_D$, m p , and mixed m p) with the di-*O*-methyl-D-mannose described by Urbas *et al* [2]

Confirmation that the crystalline product was 2,4-di-*O*-methyl-D-mannose was obtained by periodate oxidation of the corresponding di-*O*-methylhexitol (obtained

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†Tosyl = toluene-*p*-sulphonyl

by reduction with borohydride), after 4 h, 1 1 moles of oxidant per mole had been consumed, and no formic acid was produced After 24 h, these figures were unchanged, and 2,4-di-*O*-methyl-D-arabinose was then isolated from the periodate-oxidized material

In view of the structure of mannose, the periodate-oxidation results presented above, and those obtained by Urbas *et al* [2], would not distinguish between 2,4- and 3,5-di-*O*-methyl-D-mannose A distinction can, however, be drawn by bromine oxidation, when the former yields a δ-lactone and the latter a γ-lactone, the lactones are readily distinguishable by their C=O stretching absorptions in the infrared[7]

EXPERIMENTAL

The general experimental methods have been reported previously[8] Optical rotations were measured on a Perkin–Elmer 141 polarimeter

Methyl 3-O-toluene-p-sulphonyl-6-O-trityl-α-D-mannopyranoside (3). — Methyl α-D-mannopyranoside[9] (1) (20 0 g, m p. 191–192°, $[\alpha]_D^{25}$ +78 8° in water) was dissolved in dry pyridine (500 ml), and chlorotriphenylmethane (36 0 g) was added. After being kept for 48 h at room temperature, the solution was poured into ice–water, and the product was isolated as a solid which was fractionated on a column of silica gel with chloroform–acetone (85 15) The homogeneous (t l c) fractions were combined and evaporated to yield a solid (41 0 g, 73%), which failed to crystallize The purified 6-trityl ether 2 showed $[\alpha]_D^{30}$ +22 7° (*c* 5 3, chloroform); lit [10], $[\alpha]_D$ +23 4° (chloroform)

Anal Calc for $C_{26}H_{28}O_6$ C, 71 57, H, 6 42 Found C, 71.30, H, 6 16

The trityl ether 2 (38 0 g) in dry pyridine (500 ml) was esterified by the dropwise addition of a solution of tosyl chloride (25 0 g) in dry pyridine (150 ml) during 15 h at 50° After a further period of 6 h at 50°, the reaction mixture was cooled to room temperature and poured into ice–water. The product was extracted with chloroform (8 × 200 ml), and the combined extracts were washed successively with water, 10% cupric sulphate solution, and water, dried over sodium sulphate, and evaporated A portion (500 mg) of the resulting solid (49 0 g) was fractionated by t l c with chloroform–acetone (85 15), giving, in addition to the starting material (0 170 g, R_F 0 05), the following three fractions

Fraction	*Yield (mg)*	R_F	*Found S (%)*	*Formula*	*Calc S (%)*
1[a]	67	0 24 0 27	5 05	$C_{33}H_{34}O_8S$	5 42
2[b]	226	0 59	5 35	$C_{33}H_{34}O_8S$	5 42
3[c]	10	0 97	8 38	$C_{40}H_{40}O_{10}S$	8 61

[a]Mixtures of methyl 2-*O*-tosyl-6-*O*-trityl-α-D-mannopyranoside and methyl 4-*O*-tosyl-6-*O*-trityl-α-D-mannopyranoside. [b]Methyl 3-*O*-tosyl-6-*O*-trityl-α-D-mannopyranoside [c]Methyl di-*O*-tosyl-6-*O*-trityl-α-D-mannopyranoside

The mixture of toluene-*p*-sulphonyl derivatives was fractionated by column chromatography on silica gel to give a homogeneous (t l c) sample (3.5 g) of sulphonate **3**, $[\alpha]_D^{25}$ +29 6° (*c* 2 8, chloroform).

Anal Calc for $C_{33}H_{34}O_8S$· C, 67 11; H, 5 80; S, 5 42 Found. C, 66 78, H, 6 05; S, 4.96

Methyl 2,4-di-O-methyl-3-O-tosyl-6-O-trityl-α-D-mannopyranoside (**4**) — Methyl 3-*O*-tosyl-6-*O*-trityl-α-D-mannopyranoside (**3**) (3 4 g) was treated with methyl iodide (40 ml) and silver oxide (4 0 g) Filtration and evaporation gave a product which, after seven such further methylations, yielded compound **4** (3 2 g, 95%) which crystallized readily from ethyl acetate and had m p 199–201°, $[\alpha]_D^{24}$ +38 2° (*c* 2.08, chloroform)

Anal. Calc for $C_{35}H_{38}O_8S$ C, 67 94: H, 6 19; S, 5 18; OCH_3, 15 05. Found C, 67 90; H, 6 32; S, 5 15; OCH_3, 15 06

Methyl 2,4-di-O-methyl-6-O-trityl-α-D-mannopyranoside (**5**) — A solution of compound **4** (1 98 g) in dry benzene (25 ml) was mixed with a solution of sodium methoxide [from 2 5 g of sodium in methanol (50 ml)], and the mixture was refluxed on a sand bath for 9 h Water (25 ml) was added to the cooled solution, organic solvents were distilled off, and the suspension was extracted with chloroform (5 × 25 ml) The extract was dried over sodium sulphate and evaporated to a syrup (1 45 g, 97%) which was crystallised from ethanol to give the title compound, m p. 167–168°, $[\alpha]_D^{22}$ +25 2° (*c* 2 25, chloroform)

Anal Calc for $C_{28}H_{32}O_6$· C, 72 39; H, 6 94, OCH_3, 20 04. Found C, 72 30, H, 6.94; OCH_3, 19 94

Methyl 2,4-di-O-methyl-α-D-mannopyranoside (**6**) — Trityl ether **5** (1 34 g) was dissolved in glacial acetic acid (30 ml), water (13 ml) was added, and the turbid solution was stirred magnetically at room temperature. After 7 h, the precipitate was removed by filtration, the solution was evaporated, and water was distilled from the residue several times, keeping the temperature below 30°. The residue was dissolved in chloroform–acetone (1 1) and was fractionated on a column of silica gel by elution with acetone–chloroform (1 15) to remove triphenylmethanol, and then with acetone–chloroform (1 1) to remove compound **6** (0 61 g, 96%), $[\alpha]_D^{17}$ +54° (*c* 4 3, water) (Found· OCH_3, 41 3; Di-*O*-methylhexoside calc : 41 9)

2,4-Di-O-methyl-D-mannose (**7**) — Glycoside **6** (0 49 g) was heated with N sulphuric acid (10 ml) at 100°, and the hydrolysis, which was followed by t l c (chloroform–acetone, 1 1), was complete in 12 h The acid was neutralised ($BaCO_3$), and the filtered solution was evaporated to give a syrup (0 45 g, 98%) which crystallised readily Recrystallisation from ethyl acetate, containing a few drops of methanol, gave the title compound, m.p 124–125°, $[\alpha]_D^{21}$ +13 6 (3 min)→+11 4° (37 min, equil.); Urbas *et al.*[2] reported m p. 126–127°, $[\alpha]_D^{20}$ +14 5° (*c* 1 5, water) Paper chromatography and electrophoresis showed a single component having R_F 0 22, R_G 0 28 (solvent *A*), M_G (streak), and a reddish brown colour reaction with spray *A* Demethylation[11] produced mannose These values agreed with those obtained from the sample of Urbas *et al* [2] placed on the same chromatogram and electrophoretogram.

Periodate oxidation of 2,4-di-O-methyl-D-mannitol — 2,4-Di-*O*-methyl-D-mannose (7) (130 mg) was reduced with sodium borohydride[12]. The resulting, syrupy hexitol (106 mg) was oxidized in aqueous solution (100 ml) containing 0 3M sodium periodate (20 ml) The consumption of periodate determined by the arsenite method[13] was 1 1 moles per mole of hexitol after oxidation for 4 and 24 h No formic acid was produced

The solution (90 ml) remaining from periodate oxidation was concentrated, deionized with Rexyn ion-exchange resins, and evaporated. The residue was extracted with acetone–chloroform (1.1), and the soluble portion was purified by t l c with the same solvent mixture The syrupy product (23 mg) showed R_F 0 25 (solvent *A*, and reddish brown colour reaction with aniline hydrogen phthalate), M_G 0 00, and $[\alpha]_D^{17}$ −137° (*c* 2 03, water), and produced arabinose on demethylation[11] These values were in excellent agreement with those obtained for a sample of 2,4-di-*O*-methyl-D-arabinose, lit [13], $[\alpha]_D$ +129° for 2,4-di-*O*-methyl-L-arabinose

The syrupy sugar (20 mg) in ethanol (1 ml) was heated under reflux with freshly distilled aniline (12 mg) for 4 h The ether-soluble portion crystallized on seeding with 2,4-di-*O*-methyl-*N*-phenyl-D-arabinosylamine and had m p 141–143°; lit [14,15], for the L-enantiomorph, m p 145° and 142–144°

2,4-Di-O-methyl-D-mannono-1,5-lactone — A mixture of 2,4-di-*O*-methyl-D-mannose (67 mg), water (1 ml), barium carbonate (80 mg), and bromine (10 drops) was kept in the dark for 65 h Bromine was removed by aeration, the solution was acidified, and water was removed by evaporation The residue was extracted with chloroform–acetone (1 1), and the soluble portion was purified on two t l c plates with the same solvent The resulting syrup (22 mg) afforded the lactone, b p 165–170° (bath)/0 01 mm, which failed to crystallize and showed ν_{max} 1738 cm^{-1} (chloroform).

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NITROGEN-CONTAINING CARBOHYDRATE DERIVATIVES

PART XX*. IONISATION CONSTANTS OF AMINO SUGARS

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ABSTRACT

The pK_a values have been measured of seventeen amino-sugars having the methyl amino-4,6-*O*-benzylidene-deoxy-glycoside skeleton, including *O*- and *N*-methylated derivatives The results are discussed in terms of electronic and steric factors.

INTRODUCTION

During a study of the periodate oxidation of amino sugars[1], it became necessary to determine their ionisation constants in water (pK_a) Little work has appeared in the literature on this topic, and so advantage was taken of the availability of a wide variety of compounds containing the basic skeleton **1** of known conformation Amino-hydroxy, methylamino-hydroxy, and dimethylamino-hydroxy systems have been investigated, as has the effect of *O*-methylation and of change in anomeric configuration

Since this work was completed, a paper has appeared[2] on the prediction of pK_a values of amino sugars, in which the parameters for the presence of various functional groups in the sugar molecule were calculated, and a comparison was made of the calculated values with those found by experiment

Replacement of the hydrogen atoms in an isolated amino group increases the pK_a value, so that, for example, dimethylamine[3] is more basic than methylamine Introduction of a second methyl group, *i.e.*, to give trimethylamine, decreases the pK_a value This is because two competing effects are operative, *viz*, the inductive effect of the methyl group and solvation effects The balance of these two effects will, of course, vary from molecule to molecule Stevens *et al* [4] noted that *N*-methylation of methyl 4-amino-4,6-dideoxy-α-D-glucoside and -galactoside increased the basicity, whereas the *N,N*-dimethyl derivatives were less basic than the parent amino-compounds

The introduction of a hydroxyl group on the carbon atom adjacent to an

*Part XIX C B Barlow, E O Bishop, P R Carey, and R D Guthrie, *Carbohyd Res*, 9 (1969) 99
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amino group is known to lower the basicity of the amino groups[5], for example, ethylamine pK_a 10.67, and 2-aminoethanol pK_a 9 50 Svoboda *et al* [5] have studied *cis*- and *trans*-aminocycloalkanols and their *N*,*N*-dimethyl derivatives, and have discussed the variation in basicity of the amino group in terms of electronic and hydrogen-bonding effects

EXPERIMENTAL

Nitrogen was bubbled through Fieser's solution [sodium dithionite (16 g), sodium anthraquinone-2-sulphonate (0 8 g), sodium hydroxide (15 g), and water (100 ml)] to remove oxygen and carbon dioxide, and then through water[6]

The pH measurements were made with a Pye 'Dynacap' pH meter fitted with a combined glass and calomel reference electrode and a standard temperature compensator The instrument was standardised against potassium hydrogen phthalate (0.05M), pH 4.01 at 0 and 25°; and against potassium dihydrogen phosphate–disodium hydrogen phosphate (0 025M), pH 6 98 at 0° and pH 6 86 at 25° For pH measurements at 0°, the electrodes were immersed in ice–water for 1 h, and all solutions were precooled to 0° Distilled water at 0° was used to rinse the electrodes between readings. Water was the solvent used in all experiments and was freshly distilled from alkaline permanganate. Immediately before use, the water was boiled and then allowed to cool under nitrogen. Hydrochloric acid was the B D H volumetric reagent, standardised against "Analar" sodium carbonate The standard acid was delivered from a micrometer syringe fitted to a micrometer screw-gauge

The method was initially perfected and periodically checked by the determination[6] of the ionisation constants of standard compounds (sodium benzoate, glycine, and "Tris" buffer)

A solution of the amino sugar in pre-boiled water was agitated with nitrogen and placed in the thermostat The pH was noted, and the solution was titrated potentiometrically with standard hydrochloric acid (0 2N, 10 ml) The titrant was added in ten equal portions (0 1 ml), each one tenth of an equivalent, and the pH was recorded as soon as equilibrium had been reached after each addition When the titration was complete, the electrodes were washed and placed in buffer solution, and the pH was checked The total concentration of amino sugar at the half-neutralization point was 0 004M, and the ionic strength was 0.002 gram ion per litre

The ionisation constant of methyl 3-amino-4,6-*O*-benzylidene-3-deoxy-α-D-glucoside (**6**) was also determined by titration of the hydrochloride, the method was essentially that used above, but sodium hydroxide was used as titrant instead of hydrochloric acid. The result was in good agreement with that obtained by titration of the free base

Occasionally, the ionisation constants were determined by graphical interpolation of the titration curve, pH *vs* ml of titrant added, from the half-neutralization point A blank titration on water was obtained, and a correction was applied. The curves were clear, and the equivalence points were sharp

Compounds **1–8** were prepared as described previously[7] The synthesis of the others will be described in a future paper[8] The values obtained are shown in the Tables

TABLE I

ACID IONISATION CONSTANTS GIVEN BY THE EQUATION $RNH_3^+ \rightleftharpoons^{K_A} RNH_2 + H^+$, FOR THE ISOMERIC AMINO-SUGARS, AT VARIOUS TEMPERATURES

Methyl 4,6-O-benzylidene-α-D-glycosides	pK_a			
	25°	16 8°	8 2°	0°
	(±0 02)			(±0 03)
2-Amino-2-deoxy-alloside (**1**)	7 19	—	—	7.75
3-Amino-3-deoxy-alloside (**2**)	7 65	7 87	8 10	8 29
2-Amino-2-deoxy-altroside (**3**)	6 11	—	—	6 64
3-Amino-3-deoxy-altroside (**4**)	7 12	—	—	7.72
2-Amino-2-deoxy-glucoside (**5**)	7 17	—	—	7 78
3-Amino-3-deoxy-glucoside (**6**)	7.13	7 31	7 53	7.75
2-Amino-2-deoxy-mannoside (**7**)	6 61	—	—	7 14
3-Amino-3-deoxy-mannoside (**8**)	7 38	—	—	7 97
3-Amino-3-deoxy-2-*O*-methyl-altroside (**9**)	6 87	—	—	—
3-Deoxy-3-methylamino-altroside (**10**)	7 65	—	—	—
3-Deoxy-3-dimethylamino-altroside (**11**)	7 82	—	—	—
3-Amino-3-deoxy-2-*O*-methyl-glucoside (**12**)	7 05	—	—	—
3-Deoxy-3-methylamino-glucoside(**13**)	7 56	—	—	—
3-Deoxy-3-dimethylamino-glucoside (**14**)	7 42	—	—	—

TABLE II

Methyl 4,6-O-benzylidene-β-D-glycosides	pK_a (25°) (±0 02)
2-Amino-2-deoxy-altroside (**15**)	6 67
3-Amino-3-deoxy-glucoside (**16**)	6 86
3-Amino-3-deoxy-altroside (**17**)	6 00

RESULTS AND DISCUSSION

The values obtained are given in Tables I and II The pK_a values for the amino sugars can be explained reasonably in terms of the inductive effect (−I) and from the observations[2,5,9] that the *cis*-isomer of a *vicinal* amino-alcohol grouping on a cyclohexane ring is more basic than is the *trans*-isomer (*cis*-effect)

Hydroxy-amino compounds For compounds **1–8** and **15–17**, the inductive effect (−I) of the anomeric centre would be expected to have a greater effect on an amino group at C-2 than on one at C-3, and so 2-amino derivatives should be the less basic This is true for only three of the five pairs of compounds, and hence

there must be other strong effects influencing the basicity Quite striking are the values for the α-D and β-D series of altrosides, in which the order of basicity is reversed on changing the anomeric configuration

That the *cis*-isomer of a *vicinal* amino-alcohol group on a cyclohexane ring is more basic than is the *trans* isomer[2,5,9] is supported by the 3-amino sugars examined in the present study, the 3-amino-alloside **2** and -mannoside **8** being more basic than the *altro* (**4**) and *gluco* (**6**) isomers, respectively In the 2-amino series, the *manno* compound (**7**) is more basic than the *altro* compound (**3**), whereas the *allo* (**1**) and *gluco* (**7**) compounds have essentially the same basicity.

In general, those compounds with an axial amino group are the less-basic ones The 2-amino-altroside **3** is the least basic in the α-D series, most probably because the (−I) effect of the anomeric group is strongest at C-2 and the geometry of the molecule prevents hydrogen-bonding of the amino group with O-1 and O-3 The *cis* effect could then account for the greater basicity of its C-3 epimer, the *manno* compound **7**. In the 2-amino-β-D-altroside **15**, hydrogen-bonding is possible with the C-1 methoxyl group, and this presumably accounts for the greater basicity compared to the α-D anomer With reference to the other axial amines, the 3-amino-alloside **2** is very basic In some degree, this could be due to the *cis* effect, but there may be some effect, perhaps on solvation, due to the very strong 1,3-interaction present in such a compound Assuming, for the present, that such an effect is base-strengthening, it is of interest to compare the α- and β-D anomers of 3-amino-altroside (**4** and **15**, respectively), the former, in which the 1,3-effect will be operative, is much more basic than the latter

The effect of methylation of the neighbouring hydroxyl group was examined in two cases, namely compounds **9** and **12**, both *trans* systems It can be seen that the basicity was very slightly increased in each case (*cf.* ref 10)

Methylamino and dimethylamino systems Two series of compounds were prepared, namely **6**, **13**, and **14** with the α-D-*gluco* configuration, and **4**, **10**, and **11** with the α-D-*altro* configuration Mono-*N*-methylation was base-strengthening in both series, but di-*N*-methylation had different effects, it increased the basicity of the *altro* derivative but decreased the basicity of the *gluco* compound This may be another aspect of the *syn*-axial 1,3-effect commented on above

The study of this range of compounds, of widely differing stereochemistries, shows that the factors used by Inouye[2] for predicting pK_a values of amino sugars should include not only inductive effects but also involve closer consideration of the configurational variations from compound to compound

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THE ACTIVE SITE OF PORCINE-PANCREATIC ALPHA-AMYLASE: FACTORS CONTRIBUTING TO CATALYSIS*

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ABSTRACT

Quantitative studies reveal that the alpha-amylase-catalyzed cleavage of the α-D-(1→4) bonds in starch proceeds with retention of configuration The pH dependence of the enzymic activity, and of photo-oxidation in the presence and absence of chloride, and a spectrophotometric titration of the enzyme, implicate a carboxylate anion and an imidazolium cation as the catalytic residues of pancreatic alpha-amylase. Since the same groups are probably involved in the action of other alpha-amylases and even beta-amylase, which gives inversion of configuration during hydrolysis, it seems possible that both types of enzyme operate by a common mechanism A reaction mechanism involving general acid-catalysis by imidazole, and ring distortion to produce an oxycarbonium-ion intermediate, is proposed The carboxyl group may participate either as a general base or by electrostatic solvation of the intermediate, but a role as a nucleophile is not definitely excluded.

INTRODUCTION

Since the active centers of amylases have received scant attention, little is known of the mechanisms of their action As part of a program aimed at elucidating the mechanism of amylase-catalyzed hydrolysis of acetals, a comparative study of the active sites of porcine-pancreatic alpha-amylase and sweet-potato beta-amylase has been undertaken These two crystalline enzymes were selected because Freeman and Hopkins[1] reported that alpha- and beta-amylase hydrolyze the α-D-(1→4) bonds in starch with retention and inversion of configuration, respectively This conclusion was reached on the basis of qualitative experiments and recently was confirmed quantitatively for beta-amylase[2].

This conclusion was based upon a detailed study of the time course of the

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change in optical rotation of a maltotetraose solution subject to rapid hydrolysis by a massive amount of enzyme. A similar study with alpha-amylase as the hydrolytic catalyst and amylose (D P 60) as substrate indicated 83% retention of configuration[3] Because of technical difficulties and the uncertainty of computed rotations, the precision of this experiment was judged to be ±20% A more accurate assessment of the extent of retention is presented in this paper

The difference in stereochemistry accompanying cleavage prompted the speculation that the two enzymes act *via* fundamentally different reaction mechanisms[4] However, the difference in configuration of the products is also consistent with a common reaction mechanism in which the two enzymes differ in their control of the approach of a water molecule to the reaction center We propose a mechanism that is fully consistent with the latter suggestion and is also in accord with both physical-organic and enzymic data characteristic of acetal hydrolysis

It has been widely accepted that hydrolysis of acetals having aliphatic substituents does not involve solvent as a participating nucleophile and normally proceeds *via* a specific, hydronium-ion catalyzed, A-1 mechanism[5-11] By contrast, most carbohydrases (including porcine-pancreatic alpha-amylase and sweet-potato beta-amylase) exhibit typical, bell-shaped pH optima that are inconsistent with an A-1 mechanism[12]. While there are no really satisfactory models for amylase catalysis, recent papers[13,14] have speculated on a potential role for a carboxylate ion in the reaction. Therefore an investigation of the active sites of amylases is of considerable importance, not only for comparative purposes, but also to establish whether or not this particular nucleophile may be involved in catalysis

EXPERIMENTAL

Porcine-pancreatic alpha-amylase. — Three-times crystallized alpha-amylase was prepared according to Fischer and Stein[15] with the exclusion of chloride salts from all of the purification steps This enzyme preparation was used for all experiments unless specified

Standard enzyme assay — The assay for alpha-amylase was initiated by mixing 1 ml of enzyme with 2 ml of 0 375% starch solution (Linter soluble, from Merck and Co) in 0 18M phosphate buffer at pH 7 0, 0 75M in potassium chloride The reaction mixture was incubated at 25 ±0 1° for 5 to 10 min, and the hydrolysis was terminated by the addition of 1 ml of Somogyi's copper reagent (Nelson's modification)[16]. The hydrolyzates were then heated in a vigorously boiling water bath for 15 min in cappedt ubes, cooled in ice water, and 1 ml of arsenophosphomolybdate reagent was added The tubes were shaken vigorously and then diluted to 25 ml with deionized water, and the optical densities were read at 660 nm with a Zeiss PMQ II spectrophotometer against blanks containing all reagents except enzyme. The extent of hydrolysis was never allowed to exceed 10%. In an alternative method, enzyme action was terminated by addition of 4 ml of a modified Neocuproine reagent A[17,18]. Neocuproine reagent B was added and the mixtures were heated for 12 min in boiling water. After dilution to 25 ml, the optical density was measured at 450 nm

The extinction coefficient of alpha-amylase — Replicate samples of 2.00 ml containing 5.9 mg of alpha-amylase, dialyzed for 60 h against distilled water, were pipetted into platinum crucibles and dried to constant weight at 100–110°. From the known weights of the protein samples and their absorbance at 280 nm, the extinction coefficient was determined. During the dialysis less than 5% loss of activity occurred.

pH stability of alpha-amylase — A 1.4×10^{-7}M solution of alpha-amylase (chloride concentration $< 10^{-8}$M), a generous gift of Dr. L. Stewart, was incubated in 0.03M buffers at various pH values for 40 min at 25 ±0.1°. The buffers contained 4×10^{-4}M calcium acetate with or without 0.01% of bovine-serum albumin. Duplicate 0.2 ml samples of each of the enzyme solutions were withdrawn, diluted to 3 ml with 0.03M phosphate buffer at pH 7.0, and tested for enzyme activity by the standard assay. The pH of all the solutions after dilution was 7.0 ±0.1.

Effect of pH on K_m *of alpha-amylase* — The K_m values of alpha-amylase (Worthington Biochemical Corporation) at 25 ±0.1° at various pH values were determined in 0.02M pyrophosphate–acetate buffers 0.1M in sodium chloride and 0.01% in bovine-serum albumin. The starch concentration ranged from 0.025 to 1.0%. Hydrolysis was limited to less than 10% and measured by the standard assay. During the reaction period less than 3% inactivation of the enzyme occurred at extreme pH values, so that no correction was needed for inactivation of the enzyme.

Effects of pH on V_{max} *of alpha-amylase* — The pH–activity profiles of chloride-free alpha-amylase and of alpha-amylase in 0.025M potassium chloride or 0.1M sodium chloride were determined at 25 ±0.1°. The activity of the enzyme at each pH was measured by the standard assay procedure except for the necessary changes in buffer ions or chloride concentration. D-Glucose standards used at each pH allowed correction for the variation in color yield caused by the buffers. No correction for denaturation was made since a maximum of 3% inactivation occurred during the assay period. Maximum velocity, V_{max}, was calculated from the observed velocity, v_0, by Equation 1, which corrects for lack of enzyme saturation with substrate.

Stereochemistry of the products from reduced amylodextrin hydrolyzed with alpha-amylase. — Assay reagent A was prepared by adding 0.1 ml of *o*-dianisidine (10 mg/ml) in absolute methanol to 10 ml of 0.01M acetate buffer of pH 5.05. The *o*-dianisidine was recrystallized once from absolute methanol. Reagent B was prepared by dissolving about 10 mg of D-glucose oxidase (grade A, lot 43434, purchased from California Biochemical Corporation) in 10 ml of the acetate buffer and adding 50 µl (approximately 0.04 mg) of horse-radish peroxidase (grade A, lot 42388, purchased from California Biochemical Corporation). Solution B was stable for several days if refrigerated. A typical assay for β-D-glucose was initiated by adding 50 µl of the D-glucose solution, within 30 sec after preparation, to 1 ml each of reagent A, reagent B, and acetate buffer. The solution was mixed by inverting the cuvette several times, and the reaction was followed spectrophotometrically at 460 nm. A plot of optical density versus time was made and the concentration of β-D-glucose established by extrapolating the latter phase of the curve to zero time.

The stereochemistry of the hydrolysis products, produced by action of alpha-

amylase on a reduced amylodextrin having average chain-length of 22, was assessed by the coupled-enzyme assay The preparation of amylodextrin of average chain-length 22 has been described elsewhere[19]. This dextrin was reduced three successive times with sodium borohydride, purified by ion-exchange chromatography, and lyophilized[20].

The experiment was initiated by adding 2 ml of alpha-amylase (1 1 mg/ml), in acetate buffer 0 05M in potassium chloride, to 2 ml of reduced amylodextrin solution (1 mg/ml) in buffer After 1 min, a 1-ml aliquot was pipetted into a cuvette containing 1 ml of reagent A and 1 ml of reagent B The solution was mixed and the reaction followed spectrophotometrically at 460 nm The blank contained 1 ml of acetate buffer in place of the hydrolyzate. The remainder of the hydrolyzate was placed in a boiling-water bath for several min to inactivate the enzyme and mutarotate the sugars At a convenient time a 1-ml aliquot of the heated solution was assayed for β-D-glucose

$$V_{max} = v_0\left(1+\frac{K_m}{[S]}\right) \quad (1)$$

Spectrophotometric titration of alpha-amylase — Amylase solution for spectrophotometric titration was prepared by addition of 500 μl of enzyme to 500 μl of the appropriate buffer (0 02M phosphate, pyrophosphate, or carbonate) At pH values greater than 11, the enzyme concentration was halved. The optical densities of all solutions were read versus the enzyme at the same concentration, in phosphate buffer at pH 6 0, at 295 and 243 nm As soon as these readings were taken, 10 μl of 2 5M KCl was added to each cuvette and 10 μl of water added to the blanks and the measurements of optical density were repeated after mixing. The pH values of the final solutions were measured with a Beckman Zeromatic pH meter

Deuterium isotope effect — The Michaelis constants were determined as described above at pH or pD 7 0 except that a hydrolysis time of 10 min was used The D_2O used was >99% isotopically pure Stock solutions of enzyme were prepared by diluting a concentrated aqueous solution approximately 1000 times, either with H_2O or D_2O containing buffer and salts

Photo-oxidation of alpha-amylase — Methylene blue of histological grade from Fisher Scientific Company was employed as the photocatalyst A stock solution of 0 06 mg/ml in distilled water was stored in the dark. To photoinactivate the enzyme an incubation mixture was prepared by adding 1 ml of methylene blue, 2 ml of enzyme of appropriate concentration, and 2 ml of buffer The buffer was prepared by mixing 0 033M sodium acid pyrophosphate with 0 01M sodium pyrophosphate to give the desired pH (measured with a Leeds and Northrup pH meter) and a constant ionic strength of 0 1. The incubation mixture was then placed in a constant-temperature vessel at 25° and stirred with a magnetic spin-bar. Illumination was achieved by a 500-watt slide projector attached to a voltage stabilizer placed at a distance of 10 cm from the reaction vessel and focused thereon. At zero time and at regular intervals 200-μl aliquots were withdrawn, diluted to 10 ml, and stored in the dark until

assayed. All manipulations except the photo-oxidation were conducted in a dark room with the aid of a red safety lamp The 500-watt lamp was off during all of these manipulations After all of the aliquots had been withdrawn at time intervals they were assayed by the standard enzyme assay with the aid of the safety lamp

Another set of photo-oxidation experiments similar to those described above was conducted in the presence of 1.25×10^{-2}M potassium chloride.

An unweighted least-square analysis was then performed on an IBM 7040 computer to the line log (% residual activity) = $-kt+$intercept

During the assay period, periodic checks showed that the rate of photoinactivation at pH 7 did not change appreciably, so that the day-to-day results are directly comparable

RESULTS

Extinction coefficient of alpha-amylase — The extinction at 280 nm of a 1% solution of alpha-amylase in distilled water in a 1-cm light path was 22.9. This compares with values of 23.3 (ref. 21) and 24.1 (ref. 22) measured under slightly different conditions Assuming for the enzyme a molecular weight[23] of 45,000, the molar extinction, 1.05×10^5, in distilled water was calculated and was used throughout this work.

Configuration of hydrolytic products of the action of alpha-amylase. — The approach employed a coupled enzyme assay that took advantage of the specificity of D-glucose oxidase toward β-D-glucose[24] The sensitivity and precision of the assay system for β-D-glucose under the conditions used was established by measuring the color yield of various amounts of α- and β-D-glucose The results are displayed as a Beer's law plot in Fig 1. The insert illustrates the characteristic kinetics of the

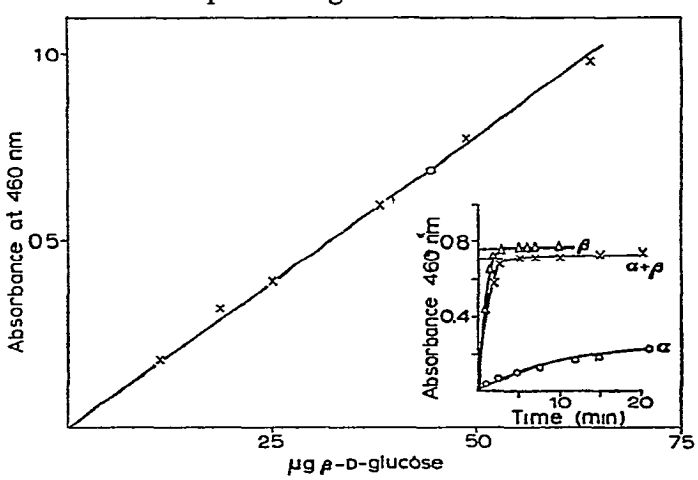


Fig 1 Beer's Law plot for β-D-glucose assayed by the D-glucose oxidase system For conditions consult text ×, β-D-glucose, ○, mixture of β-D-glucose (43 μg) and α-D-glucose (14 μg)

Insert time course of oxidation ○, α-D-glucose (50 μg), △, β-D-glucose (48 μg), ×, mixture of β-D-glucose (43 μg) and α-D-glucose (14 μg) The open circle on the Beer's law plot corresponds to the extrapolated point in the insert for a mixture of α- and β-D-glucose

color production, and the plot demonstrates the remarkable specificity of D-glucose oxidase With this information at hand, a study of the configuration of the products of amylase hydrolysis was undertaken.

Although β-D-glucose can be detected in substantial amounts in a boiled and mutarotated hydrolyzate, none was detected immediately subsequent to hydrolysis This conclusion is evident by inspection of Fig. 2, which shows a marked difference

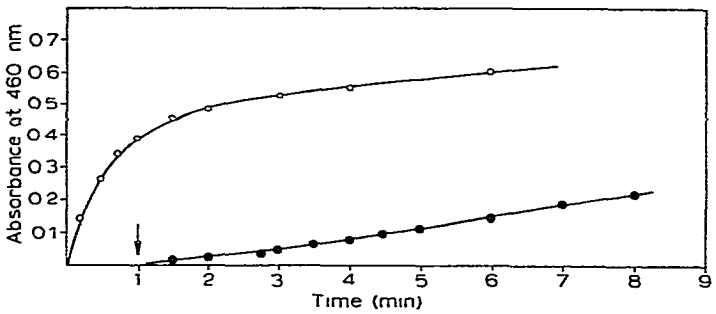


Fig 2 Time course of oxidation of β-D-glucose in alpha-amylase hydrolyzate of reduced amylodextrin O, approximately 1 min after addition of alpha-amylase to amylodextrin a sample was heated to destroy enzymic activity and mutarotate sugars An aliquot was then assayed for β-D-glucose Zero time corresponds to addition of a heated aliquot to D-glucose oxidase ●, hydrolyzed sample submitted to D-glucose oxidase 1 min (arrow) after addition of alpha-amylase (zero time) For additional conditions consult text

in the kinetics of color production for a heated and unheated alpha-amylase hydrolyzate of reduced amylodextrin If 2–3% of the D-glucose had been of the β-configuration, it should have been detected by a small but rapid increase in color in the first few min of reaction Therefore its absence allows us to conclude that alpha-amylase achieves essentially complete retention of configuration during hydrolysis A chromatographic analysis[25] of the reaction mixture taken a few min after addition of enzymes (boiled to stop enzyme action) revealed that D-glucose comprised about 20% of the mixture, the remainder being mainly maltose and maltotriose. Trace amounts of larger, branched saccharides were also detected The lower curve in Fig 2 is slightly concave upward because the maltotriose was cleaved slowly to yield α-D-glucose, which mutarotates and is then oxidized Strictly speaking, it has only been shown that D-glucose has retained its anomeric configuration However, when a reduced substrate was used the reducing end of D-glucose could only have arisen from a central bond that must have been cleaved some time during hydrolysis It is highly probable, therefore, that all products must have retained their anomeric configuration

pH stability of alpha-amylase. — Alpha-amylase is stable in the absence of chloride over most of the pH range 4–10 5 Significant inactivation was detected during a 40-min incubation period (see Table I) only at the extremes of the pH range. The stability was not significantly altered in the presence of 0 01% bovine-serum albumin. Under standard assay conditions at the extremes of pH, less than 3%

inactivation of the enzyme could have occurred The chloride-activated enzyme is also completely stable, even at the extreme pH values used in the assay[3,26]

TABLE I

PERCENTAGE OF REMAINING ACTIVITY OF CHLORIDE-FREE ALPHA-AMYLASE AFTER INCUBATION FOR 40 MIN AT DIFFERENT pH VALUES[a]

pH	*Remaining activity[b], percent*
3 91	85
5 20	98
5 92	98
6 90	100
8 20	96
9 34	94
9 98	99
10 45	85

[a]The chloride-activated enzyme has been shown to be completely stable during the assay period at all pH values employed [b]The presence of 0 01% bovine-serum albumin did not significantly increase enzyme stability

Effect of pH on K_m *of alpha-amylase* — The double reciprocal plots of 1/velocity versus 1/[substrate] were linear, within experimental error, at all pH values The dependence of pK_m on pH is shown in Fig 3 The breaks in the curve, presumably characteristic of the ionizing constants of binding amino acids[27], occur at approximately pH 5 2 and 8 7. The slopes of the acid (0 8 ±0 1) and alkaline (0 95 ±0 1)

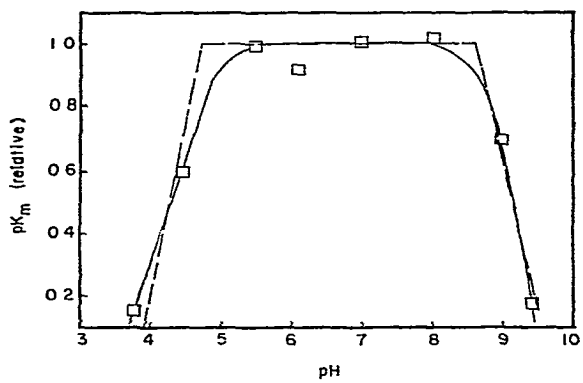


Fig 3 pH dependence of $-\log \tilde{K}_m$ of starch–alpha-amylase system Velocity measurements were made under standard assay conditions except for adjustment of pH with pyrophosphate–acetate buffer and presence of 0 01% bovine-serum albumin in 0 1M chloride Straight dashed lines represent theoretical slopes of zero or ± unity.

arms of the curve are approximately unity, suggesting that a single acidic and a single basic group on the protein govern substrate binding A more critical interpretation of these constants is given below.

Effect of pH *on* V_{max} *of alpha-amylase.* — The variation of the apparent maximum velocity of alpha-amylase action with pH is shown in Fig 4 The pH–activity profile in 0 1M sodium chloride was superimposable on the profile in 0 025M potassium chloride

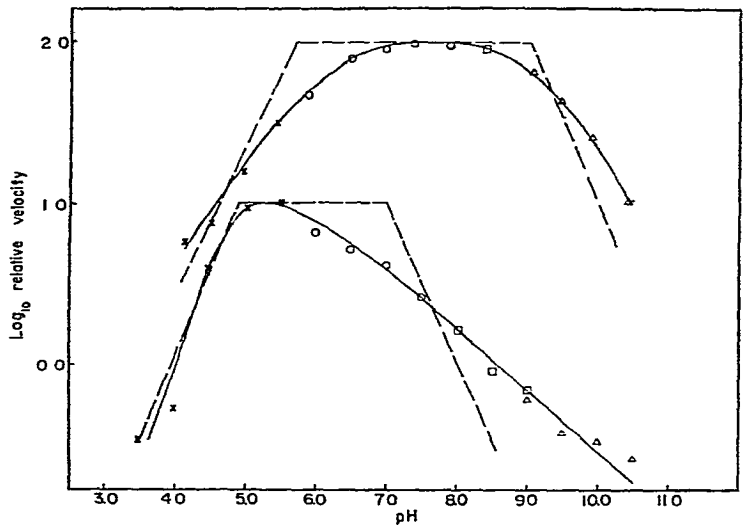
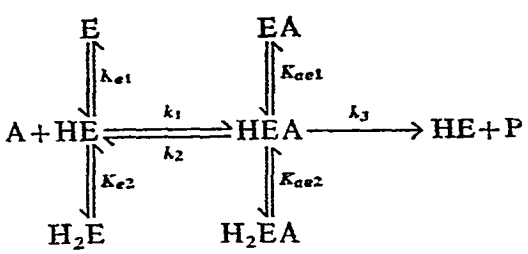


Fig 4 pH dependence of velocity of alpha-amylase at saturating starch concentration in the presence and absence of chloride ion Upper curve, 0 025M KCl at 25°, lower curve, absence of chloride at 25° ×, acetate, ○, phosphate, □, pyrophosphate, △, carbonate buffers The velocities were measured sequentially at high substrate concentration and the maximum velocities computed by Equation 1 Straight dashed lines represent limiting theoretical slopes of zero or ± unity

The slopes of the acidic and basic limbs of the curves in Fig 4 are less than unity, indicating that the simple model of the pH dependence[27] of enzyme action does not describe adequately the pancreatic alpha-amylase–starch system The divergence from unit slopes may arise from electrostatic perturbation of ionization constants, multiple intermediates, or conformational effects[28–30] The simple, two-proton model (Scheme I)[28–30] is incapable of explaining a simultaneous, bell-shaped dependence of V_{max} and K_m



Scheme I Two-proton model to explain the pH dependence of enzyme action

According to the analysis of Scheme I

$$\tilde{v}_0 = \frac{\tilde{V}[A]}{\tilde{K}_m + [A]} \qquad (2)$$

$$\tilde{V}_{max} = \frac{\tilde{V}}{1+K_{ea1}/H^+ + H^+/K_{ea2}} \quad (3)$$

$$\tilde{K}_m = \frac{K_m(1+K_{e1}/H^+ + H^+/K_{e2})}{1+K_{ea1}/H^+ + H^+/K_{ae2}} \quad (4)$$

$$\frac{\tilde{K}_m}{\tilde{V}_{max}} = \frac{K_m}{V}\left(1 + \frac{K_{e1}}{H^+} + \frac{H^+}{K_{e2}}\right) \quad (5)$$

(where the wavy line implies apparent constants; V and K_m are the pH-independent, Michaelis constants; the subscripts *e* and *ea* denote properties of the enzyme and intermediates, respectively; and the subscripts *1* and *2* correspond to acid and basic dissociation constants, respectively). It is quite obvious after assigning reasonable values to the dissociation constants that the curves corresponding to Eqs *3* and *4* cannot have the same shape. In order to account for the observations with alpha-amylase it is necessary to invoke a four-proton model in which one set of protons is associated with a pair of catalytic residues and the other set is associated with a pair of dissociable amino acids required for attachment of substrate This requires the addition of Eqs. *6–8* to Scheme I.

$$E \overset{K_3}{\rightleftharpoons} EH \overset{K_4}{\rightleftharpoons} EH_2 \quad (6)$$

$$HE \overset{K_3}{\rightleftharpoons} HEH \overset{K_4}{\rightleftharpoons} HEH_2 \quad (7)$$

$$H_2E \overset{K_3}{\rightleftharpoons} H_2EH \overset{K_4}{\rightleftharpoons} H_2EH_2 \quad (8)$$

In this set of equations, the protons on the right are associated exclusively with binding and those protons on the left are associated exclusively with catalysis Since the catalytic groups are not involved in binding, it is reasonable to expect that $K_a \simeq K_{ae}$ If substrate only combines with the enzyme forms given in Equation *7*, then the familiar Michaelis–Menten relationship is found to be·

$$\tilde{v}_0 = \frac{V/\gamma\,[A]}{K_m\alpha + [A]} \quad (9)$$

and

$$\tilde{V}_{max} = \frac{V}{\gamma} = \frac{V}{1+K'_{ae1}/H^+ + H^+/K'_{ae2}} \quad (10)$$

$$\tilde{K}_m = K_m\alpha = K_m(1+K_3/H^+ + H^+/K_4) \quad (11)$$

$$\frac{\tilde{K}_m}{\tilde{V}_{max}} = \frac{K_m}{V}(1+K_3/H^+ + H^+/K_4)(1+K'_{ae1}/H^+ + H^+/K'_{ae2}) \quad (12)$$

For this oversimplified situation, theory predicts $\tilde{V}_{max}$ and $\tilde{K}_m$ both give bell-shaped curves However, when multiple intermediates are included in the scheme, the inflection points in the $\tilde{V}_{max}$ and $\tilde{K}_m$ curves will be weighted averages of dissociation constants, and their significance is difficult to specify The difficulties inherent in the interpretation of experimental data in terms of the two-proton model are also associated with the more elaborate model. Because of the complexity of the model it is no longer possible to measure true equilibrium constants Assuming that complexing with the substrate does not greatly perturb the ionization constants of the enzyme, equilibrium constants can be approximated from the log $\tilde{V}_{max}$ versus pH profiles The pK_a values in the presence and absence of chloride are estimated to be 5 6–5.9 and 4.7–4.9, respectively. The pK_b values in the presence and absence of chloride are estimated to be 8 8–9 0 and 6 0–6 4, respectively Some of the shift in the pH–rate profile upon addition of chloride may arise from a change in the rate-limiting step.

Removal of chloride ion from alpha-amylase diminishes activity and lowers the pK values of both catalytic groups This shift is anticipated for the removal of a negative ion from the proximity of dissociable groups[31] The larger perturbation of pK_b by chloride suggests that the anion resides nearer the basic group than the acidic group.

Phosphate content of alpha-amylase — Within experimental error no phosphate was detected in the preparation by using Bartlett's[32] procedure for total phosphate From estimates of the experimental error, it could be calculated that the phosphate content could not exceed 0 05 mole per mole of enzyme

Spectrophotometric titration of alpha-amylase — The extent of ionization of the tyrosine side-chains of alpha-amylase as a function of pH is portrayed in Fig 5. The readings were completed within 2–10 min after preparation of the samples and

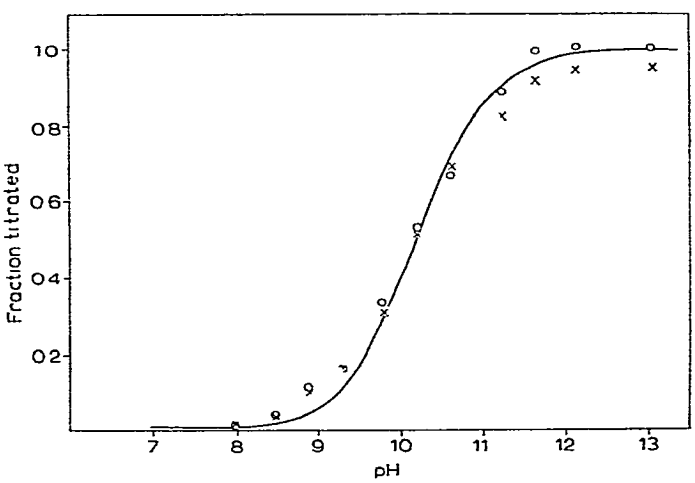


Fig 5 Spectrophotometric titration of porcine alpha-amylase with base O, 0 025M KCl, measured at 295 nm, ×, no KCl, measured at 243 nm Reference for complete ionization was 0 5M KOH

approximate equilibrium values Above pH 12 the optical density changed slowly with time (less than 10% within 10–15 min) Below pH 12 the readings were stable with time The excellent agreement between the curves measured at 295 and 243 nm below pH 11 is evidence that only spectral changes contributing to the titration curve are associated with the ionization of tyrosine residues The experimental curves at both 295 and 243 nm (Fig 5) correspond closely to a theoretical titration-curve characteristic of a monobasic acid having a pK_a of 10 2 Deviations of the experimental points from the calculated titration curve in the pH range of 8–10 could arise from numerous causes[33] Our purpose here is not to attempt an explanation of the deviation but to exclude tyrosine as a catalytic residue The enzyme in the presence or absence of chloride exhibits no detectable ionization of phenolic side chains below pH 8, which is approx. 1 2 pK units above the pK value for the basic group (as estimated by $\bar{V}_{max}$–pH dependence or photo-oxidation–pH dependence) Furthermore, ionization shifts are caused by addition of halide ion (when $\bar{V}_{max}$ and photo-oxidation rates versus pH are used as an index), but no shifts below pH 11 are observed spectrophotometrically Since the enzyme is reported to contain 13 tyrosine residues[15] and is probably a dimer[34], titration of two phenolic groups (15% ionization) should be readily detectable Thus, the failure to observe ionization of phenolic groups below pH 8 in the absence of chloride very likely precludes their involvement in catalysis

Photo-oxidation of alpha-amylase — The elegant work of Westhead[35] and others[36] demonstrated that, in the pH region of 6–9, only imidazole of the sensitive protein side chains shows a pH-dependent rate of oxidation. This simple finding

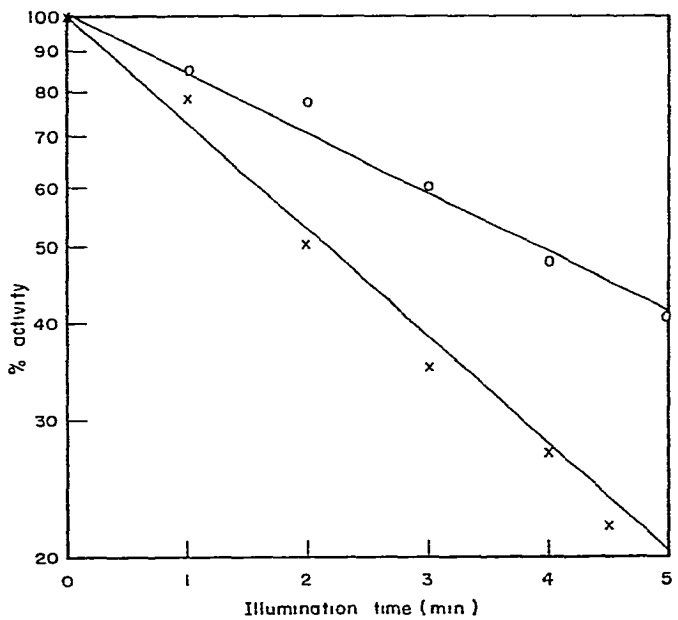


Fig 6 Typical plots for photo-oxidation of alpha-amylase employing methylene blue as the catalyst O, pH = 7 0, ×, pH = 8 5

has greatly enhanced the utility of this technique as a means of implicating catalytic amino acids in enzymes Typical time-course curves of photocatalyzed inactivation of alpha-amylase are displayed in Fig 6 In other runs not shown it was found that inactivation was pseudo-first-order with respect to time, to at least 85% inactivation Plots of this type were used to estimate how the rate changed with pH The results of this study in the presence and absence of chloride are recorded in Fig 7. Because of the multiple equilibria associated with photo-oxidation[36] it is likely that the sigmoidal curves in Fig 7 give only apparent ionization constants Nevertheless, the apparent pK value in the absence of chloride is quite typical of imidazole. As would be expected on the basis of electrostatic effects, the apparent pK increases upon addition of chloride; the increase is about 1 pK unit. We conclude that histidine is almost certainly involved in catalysis

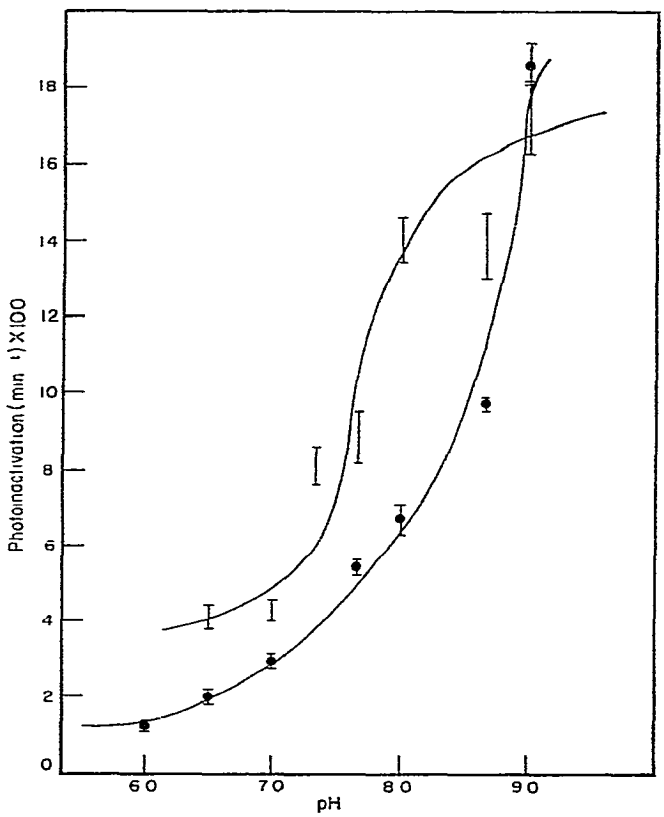


Fig 7 Effect of pH on the rate of photoinactivation of alpha-amylase I, absence of chloride, ∓ presence of 0 0125M chloride

It is interesting that the apparent pK values as evaluated from Fig. 4 seem grossly perturbed from normal values Two explanations may be offered for this phenomenon Firstly the pK values derived from $\bar{V}_{max}$–pH dependence are associated

with the enzyme–substrate intermediates and not the free enzyme. Secondly, the apparent constants are weighted dissociation constants and are influenced by rate as well as by thermodynamic factors Thus, activation by halides would be expected to cause shifts in pK values by changing relative rate-constants, even if hydrogen-ion equilibria are not shifted.

Deuterium isotope effect — $\tilde{K}_m$ and $\tilde{V}_{max}$ values at a pH or pD of 7.0 at 25° in KCl were measured as described above, and were calculated by the unweighted least-square fit to a hyperbola. The ratios, $\tilde{K}_mH_2O/\tilde{K}_mD_2O$ and $\tilde{V}_{max}H_2O/\tilde{V}_{max}D_2O$ were found to be 1 3 and 1 26, respectively Changes in the intrinsic ionization constants of the catalytic residues upon changing from H_2O to D_2O could conceivably influence the $\tilde{V}_{max}H_2O/\tilde{V}_{max}D_2O$ ratio[37]. Michaelis constants were evaluated on the plateau portion of the curve, so that the reported ratio would not differ by more than a few per cent from the ratio characteristic of the enzymes of identical ionic form in the two solvents What alterations in the conformation of the protein, if any, occur upon transfer from H_2O to D_2O, are unknown

DISCUSSION

Catalytic amino acids — Native porcine-pancreatic alpha-amylase is not inhibited by sulfhydryl reagents[15], but the enzyme does contain masked sulfhydryl groups that can be exposed by partial denaturation of the enzyme[38]. On the basis of this evidence, it has been accepted that the sulfhydryl groups are not mandatory for activity Since phosphate is not present in the enzyme, it can be excluded as a catalytic agent Tyrosine can probably be excluded by the spectrophotometric titration curve of the protein (see Results)

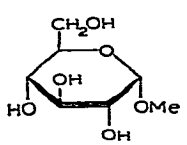
Porcine-pancreatic amylase prepared by precipitation with glycogen is acylated at the amino terminal[39]. The enzyme prepared according to a modified Fischer and Stein technique, although fully active[40–42], possesses a variable content of α-amino groups probably because of limited proteolysis An α-amino group is thereby excluded as a catalytic group Strong support that the basic group is imidazole comes from the pH dependence of photo-oxidation. Although these data do not distinguish between a binding and catalytic role for imidazole, we favor the latter possibility for the following reason Both $\tilde{V}_{max}$ and the rate of photoinactivation are strongly dependent upon the level of chloride, whereas $\tilde{K}_m$ is not (work in progress) On the basis of the photo-oxidation studies and spectrophotometric and pH investigations, carboxyl and imidazole side-chains are tentatively assigned as the groups involved as the catalytic amino acids Reasons for favoring the carboxylate anion and imidazolium cation as the active species, rather than the kinetically equivalent uncharged groups, have been presented in an earlier publication[12] Interestingly, beta-amylase appears to employ these same residues to promote hydrolysis[43,44]

Earlier, Koshland[4] suggested that, by analogy with model organic reactions, it would be expected that enzymes like alpha-amylase, which act with retention of configuration, would operate by covalent catalysis or a double-displacement mecha-

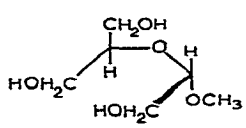
nism. By contrast, enzymes like beta-amylase, which cause inversion of stereochemistry at the reaction center, would operate by a single-displacement mechanism This hypothesis appears quite sound if the attacking nucleophile plays a major role in forcing the substrate along the reaction profile toward the transition state In this case the logical direction of attack is from the back side On the other hand, if the *C1* conformation of a D-glucosyl residue were distorted upon binding, into a half-chair conformation, ball and stick models[12] reveal that steric hindrance to a front-side attack by water would be greatly relieved In fact steric restrictions for a front-side attack by water are quite similar to those encountered for a rear-side approach The models also indicate that, if an oxycarbonium ion is formed as an intermediate, a very small change in the direction of the approaching water molecule would be required to change from retention to inversion of configuration If, as the X-ray analysis of lysozyme[46] suggests, the ring undergoing hydrolysis is in a half-chair conformation, Koshland's arguments based on small unstrained model compounds lose much of their force

On the basis of existing information, it certainly is reasonable to speculate that both alpha- and beta-amylase promote single-displacement reactions Stereochemical control of anomeric configuration might simply be governed by the direction of approach permitted to the approaching water molecule Acceptance of the similarity in the mechanism of action of alpha- and beta-amylase requires that the deuterium isotope-effect on $\tilde{V}_{max}$ be the same It is observed that (within experimental error) the ratio, $\tilde{V}_{max}H_2O/\tilde{V}_{max}D_2O$ is 1 25 for both enzymes[43] Other workers[47] have examined this ratio as a function of pH Their results at neutral pH are in substantial agreement with ours The constancy of this ratio does not necessarily support a rate-determining proton transfer[48,49] but is consistent with it and is inconsistent with a pre-equilibrium proton transfer preceding the rate-determining step

Although interest in enzyme-induced strain has again come into vogue, very little effort has been expended to determine quantitatively the extent to which strain in the substrate may enhance the catalytic process Because a substantial number of nonbonded interactions and torsional strains are introduced into a glucopyranoside when it is twisted into the half-chair conformation[50] (a conformation approaching the transition state for hydrolysis), hydrolysis of these compounds is quite sluggish compared with structurally similar open-chain acetals The magnitude of this strain effect on rates of hydrolysis can be estimated by comparing the hydrolysis rate of methyl α-D-glucopyranoside (**1**) with that of an open-chain analog (**2**)



1



From the energies of activation for acid-catalyzed hydrolysis, it can be computed[50] that **2** is cleaved 10^6 times faster than **1** at 25°. By forcing the pyranose

ring toward the half-chair form, the enzyme could accelerate the reaction by a factor of $\sim 10^6$ One other advantage that could accrue by twisting the ring is the introduction of additional bond-angle strain at the reaction center In the strained conformation the C-2–C-1–O-5 angle would be enlarged One might therefore expect a decrease in the p character of these two bonds and an increase in the p character of the H-1–C-1–O-1 bonds Concomitant with elongation, weakening of the C-1–O-1 bond would then result Schleyer[51] has reported that bond-angle strain may lead to enormous changes in reaction rates Differences of many orders of magnitude were encountered for solvolysis rates for the systems studied[51]. Schleyer's compounds ranged from rather flexible rings to rigidly constrained ones Although it is impossible to calculate quantitatively what acceleration might result from this effect in hydrolysis of acetals, it appears that it could be significant According to the transition theory, a rate enhancement of 10^6 is achieved by lowering the energy difference between the ground state and transition state by ~ 8.7 kcal mole^{-1}. This energy expenditure, which must be sacrificed during enzyme–substrate association, seems inordinately large. Substrate distortion is more likely, then, to be only a contributing factor rather than the major driving force accounting for rate acceleration.

Model studies by Fife[52,53] have revealed that, as better leaving groups are substituted at the acetal reaction center, solvent participation in the transition state is enhanced and the reaction shifts away from an A-1 mechanism. In fact, either steric or electronic factors can cause a shift from a specific acid-catalyzed, A-1 reaction to a general acid-catalyzed reaction. It appears reasonable then to speculate that ring distortion and elongation of the C-1–O-1 bond may permit the normally inert aryl acetal to become sensitive to general acid-catalysis

The most probable role for the protonated imidazole group is that of a general acid. Since the rate of acetal hydrolysis is profoundly influenced by polar effects[54], formation of a hydrogen bond to the acyclic oxygen atom would aid in polarizing the C-1–O-1 bond Even though it is impossible to quantitate this effect for acetal catalysis, it is pertinent to report that σ^*, the polar-series reaction-constant, is quite large (-3.6)

The mode of participation of a carboxyl group is difficult to specify. It may function as a nucleophile, a general base, or be involved in electrostatic solvation of a carbonium ion The simplest formulation of amylase-catalyzed hydrolysis of a glycoside is a single-displacement reaction For this mechanism, the most logical involvement of a carboxyl group would be either to assist the ionization of the attacking water molecule or to stabilize with its electric field the developing oxy-carbonium ion In either case the localization of the charge in a hydrophobic medium should increase its effectiveness

Earlier[43] it was proposed that ion-pair formation was a major contributing factor in beta-amylase catalysis The work of Cram[55] and Winstein[56], who reported dramatic accelerating effects for certain reactions in non-polar solvents in the presence of salts, was presented as supporting evidence Some experimental data relevant to this proposal have been reported by several workers[57–59]. They examined the effects

of various mineral acids and organic solvents (to vary the dielectric constant) on the rate of specific acid-catalyzed hydrolysis of acetals Reaction rates were remarkably insensitive to changes in dielectric constant between 20 and 100, they varied by only two orders of magnitude Perchloric acid was an exception, and rate changes of 10^4 were observed. It is difficult to specify precisely the reasons for the trends, because the rates are influenced not only by solvent basicity and dielectric constant but also by the extent of mineral-acid association and the extent of protonation of the acetal However, it does seem safe to assume on the basis of these data that the solvent polarity, and thus ion-pair formation, is not the major contributing factor to amylase catalysis Koshland and Neet intuitively arrived at the same conclusion[60]. These arguments of course do not exclude electrostatic solvation of an oxycarbonium ion by a carboxylate anion but only preclude this effect as the dominant factor contributing to the catalytic power of amylases

In an earlier publication[12] it was estimated, assuming only that proximity and orientation effects were important, that the maximum pseudo-first-order rate constant for amylase catalysis by a carboxyl and imidazole group was about 0 5 sec^{-1}, a number approximately 10^5 times smaller than the observed catalytic coefficients for amylases. If a factor of 10^5 is generously allowed for relief of nonbonded interactions and torsional strain, and another factor of 10^2 allowed for additional bond-angle strain when the enzyme binds the substrate, the maximum calculated rate-constant for enzyme-promoted catalysis can be accounted for It is unfortunate, though, that only a maximum value for the rate constant for the enzyme may be computed If in fact this rate is a gross overestimate of the actual enzyme-catalyzed rate, additional factors responsible for amylase efficiency remain to be explained Quite obviously, more extensive studies on model systems are needed for more critical comparisons of enzyme-catalyzed and non-enzyme-catalyzed rates of acetal hydrolysis

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THE INFRARED SPECTRA OF CARBOHYDRATES AT SUB-AMBIENT TEMPERATURES

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ABSTRACT

The infrared spectra of a large number of carbohydrates, and of several other compounds of biological interest, have been recorded, for the range 2000–250 cm^{-1}, at ~ 110°K. In addition, the i r. spectra of some selected sugars have been recorded over the range 4000–33 cm^{-1} at temperatures down to 20°K The spectra of these compounds near 110°K, conveniently attained with boiling nitrogen, are shown to be of much better quality than those obtained at room temperature, and the spectral changes observed are much greater than those usually observed on cooling simpler compounds Hence, the technique is useful for identification, characterization, and differentiation of complex compounds of biological interest

Several possible reasons for the results observed are considered. Although the effects of temperature cannot yet be quantitatively explained, they are apparently associated with hydrogen-bonding phenomena.

INTRODUCTION

In a preliminary communication[1], we recently reported that the infrared (i.r.) spectra of sugars are very much improved at temperatures near that of boiling nitrogen (~ −196°C, ~110°K) We now give the details of this and other work on the i.r. spectra of a number of carbohydrates at ~110°K, as compared with the spectra at room temperature (~25°C, ~298°K).

The temperature dependence of the i r. spectra of solids has not been widely studied, although there have been a few thorough studies with simple compounds. In general, if no change in phase occurs, on lowering the temperature the effects noted are a slight narrowing of the absorption bands, a slight increase in the intensity of bands, and, sometimes, a certain amount of band splitting[2]. With the exception of the band splitting, these effects are, although noticeable, not striking In 1950, Hainer and King[3] reported the effect of temperature on a small region of the i r. spectrum of cholesterol, and suggested that studies of the i r. spectra of complex

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materials at low temperature might prove useful More recently, Zhbankov *et al*[4] examined parts of the i r spectra of several selected sugars at the temperature of boiling nitrogen, and noted some interesting effects, but they did not make a thorough study of the entire spectra Both of these studies were restricted to a region of the spectrum (1500–1400 cm^{-1}) in which the temperature effects are not so great as in other regions

We have now performed a study of the low-temperature i r. spectra of a large number of carbohydrates, and of several other biologically important compounds*, over wide regions of the i.r. spectrum, and have examined the complete i r spectrum (4000–33 cm^{-1}) of several compounds at various low temperatures The results indicate that use of a low temperature greatly increases the utility of i r spectroscopy for the identification and differentiation of carbohydrates and other biologically important molecules, particularly if they are hydrogen-bonded

EXPERIMENTAL

All of the samples studied were obtained from commercial sources The spectra were usually recorded for Nujol mulls of the samples, although a few were recorded for potassium bromide pellets, but no special techniques for preparation were employed. For recording the spectra at low temperatures, the concentration of the mulls was usually lower than that needed for obtaining adequate spectra at room temperature The appropriate concentration is, however, a function both of the specific compound under investigation and of the frequency range chosen for study. In general, the low-temperature spectra of carbohydrates over the range 2000–250 cm^{-1} require about two-thirds the concentration of compound needed for recording their spectra at room temperature

Spectra for the range of 298–110 °K were obtained with a Perkin–Elmer Model 421 infrared spectrophotometer over the range of 4000–250 cm^{-1}, and with a Beckman IR-11 infrared spectrophotometer over the range of 350–33 cm^{-1}. The spectra at 20 °K were recorded by Professor E R Lippincott (University of Maryland) with a Perkin–Elmer Model 621 infrared spectrophotometer equipped with a gas cryostat (Air Products Company)

Most of the low-temperature spectra were recorded near the temperature of boiling nitrogen, and were obtained by use of a low-temperature cell** constructed in our laboratories The cell consisted essentially of a stainless-steel Dewar flask whose bottom was connected to a copper block that had provisions for mounting a sample between two windows of alkali halide. The Dewar flask and the cell were enclosed in an evacuable, stainless-steel outer-jacket that bore two windows of

*Including L-carnosine, cellulose (crystalline), cholesterol, daunomycinone, glycine, serotonin creatinine sulfate monohydrate, and sodium creatine phosphate hexahydrate

**Obtainable from Beckman Instruments, Inc , Scientific and Process Instruments Division, Fullerton, California 92634, U S A.

alkali halide for passage of the infrared beam The two portions of the cell were sealed with an O-ring seal, and were bolted together after the sample had been mounted A thermocouple could be mounted in one of the windows of the sample-cell, and the leads therefrom were passed through vacuum-tight seals to a potentiometer for monitoring of the temperature Windows of cesium iodide were usually used, but any other material that is suitably transparent may be employed; when far-infrared spectra were to be recorded, the same cell was used with windows of high-density polyethylene. A pellet of alkali halide, suitably gasketed with copper washers, may, if desired, be used instead of the sample windows and sample. If the sample is a liquid at room temperature, it may be placed between two windows, with or without a spacer, just as with any other demountable cell. The same procedure may be used for Nujol mulls.

Liquid nitrogen, or other coolant, was added to the Dewar flask, and, as the temperature dropped, the cell was evacuated by means of an oil vacuum-pump; this procedure is necessary, not only to conserve coolant, but also to prevent condensation of moisture on the outer windows

X-ray diffraction data were obtained with a General Electric XRD-3 diffractometer equipped with a Geiger counter detector. Nickel-filtered radiation from copper (λ 154 18 picometers) was used for recording the diffraction patterns Low temperatures were achieved with a cold-cell especially constructed in our laboratories; it consisted of a brass specimen-mount attached to a cold finger that extended from a Dewar flask and that was cooled, by conduction, with liquid nitrogen. The specimen mount was housed in a chamber equipped with a beryllium window that passed both the incident and the diffracted beam. The chamber could be evacuated, or it could be purged with a dry, inert gas Adjustments were provided for precise alignment of the specimen in the incident beam The stability of the alignment at sub-ambient temperatures was demonstrated to be $\pm 0\,01°$ (2θ) by the use of magnesium oxide as a standard. At a pressure of 100 mtorr, a temperature of $-167\,°C$ (106 °K) (thermocouple in the specimen holder) was readily maintained

RESULTS

Infrared spectra. — A typical result, as obtained without cooling, is shown in Fig 1(A), which shows the i r spectrum in the 2000–250 cm^{-1} region of a Nujol mull of α,α-trehalose dihydrate at room temperature The spectrum is typical of that obtained with the higher sugars, especially those studied as hydrates The strong band at 1680 cm^{-1} is due to water of hydration, which also contributes to the broad, ill-defined band near 700 cm^{-1} The only region of the spectrum that is fairly well defined and, therefore, useful for identification is that between 1450 and 800 cm^{-1}, indeed, part of this region is not particularly useful This is the region that has been examined in much of the previous work on the i r spectral identification and characterization of sugars[5] More recently, there has been some interest in the spectra of simple sugars[6] in the lower-frequency region (700–250 cm^{-1}), but it is

obvious from Fig 1(A) that spectra–structure correlation, and identification, of sugars by use of this region is difficult

The spectrum in Fig 1 (B) shows the effect of cooling this compound to 110°K. Again, the spectrum is for a Nujol mull, but the concentration is about two thirds of that for the room-temperature spectrum. The two traces represent two different path-lengths The quality of the spectrum is greatly improved overall, and the most striking results are at the lower frequencies It is apparent that, for identification, much more confidence can now be placed in absorption bands in this region It should be noted that this compound contains about 10% of water. Spectra of good quality are difficult to obtain at room temperature when water is present to this extent At low temperature, however, its effect on the spectrum is greatly lessened

Fig 2 shows the results obtained with α,α-trehalose dihydrate in a potassium bromide pellet The pellet has approximately the concentration of sugar for a satisfactory spectrum at 25°C, but it is obviously too concentrated to provide a useful spectrum at low temperature This Figure, however, gives an indication of the degree of band intensification observed when the temperature is lowered The two spectra are essentially identical with those observed with the Nujol mull, but the KBr technique can lead to difficulties In some cases, poor results have been obtained, in that the spectra are not always reproducible at low temperatures, this is probably due to the sensitive nature of many of these compounds When a KBr pellet is pressed, local temperatures that are rather high are obtained; these tend to degrade sensitive materials slightly. Although the degree of degradation is not sufficiently great to cause significant changes in the room-temperature spectrum, the low-temperature spectrum is much more drastically affected. A large part of this effect is probably caused by disruption of the crystallinity of the material Although the degree of crystallinity does not seem to affect the room-temperature spectra greatly (perhaps because they are of low quality originally), it affects the low-temperature spectra to a marked degree If significant spectral improvement is to be obtained on cooling, the material must be crystalline

Fig 3 shows the spectra of a *ca* 1 1 mixture of D-glucose and D-fructose at room temperature and at about 110°K The low-temperature spectrum is, again, much the better resolved, and most of the bands of each component can be readily identified. This kind of result can only be obtained with the simple sugars, however; mixtures of the higher sugars give somewhat poorer results, as might be expected

One important feature of the temperature-dependence of the i r spectra of these compounds is that it is continuous Spectra taken at ~298°K, ~275°K, ~200°K, and ~110°K form a continuous series in which no large differences appear between any two temperatures adjacent in this series Although a temperature of 110°K is usually sufficient to bring about most of the spectral change, and is experimentally convenient, the changes occurring do not cease at this temperature Fig. 4 shows a portion of the i r spectrum of a Nujol mull of lactose monohydrate at ~298°K, ~100°K, and ~20°K (temperature of boiling hydrogen) The typical changes seen earlier are also observed with this compound the broad background

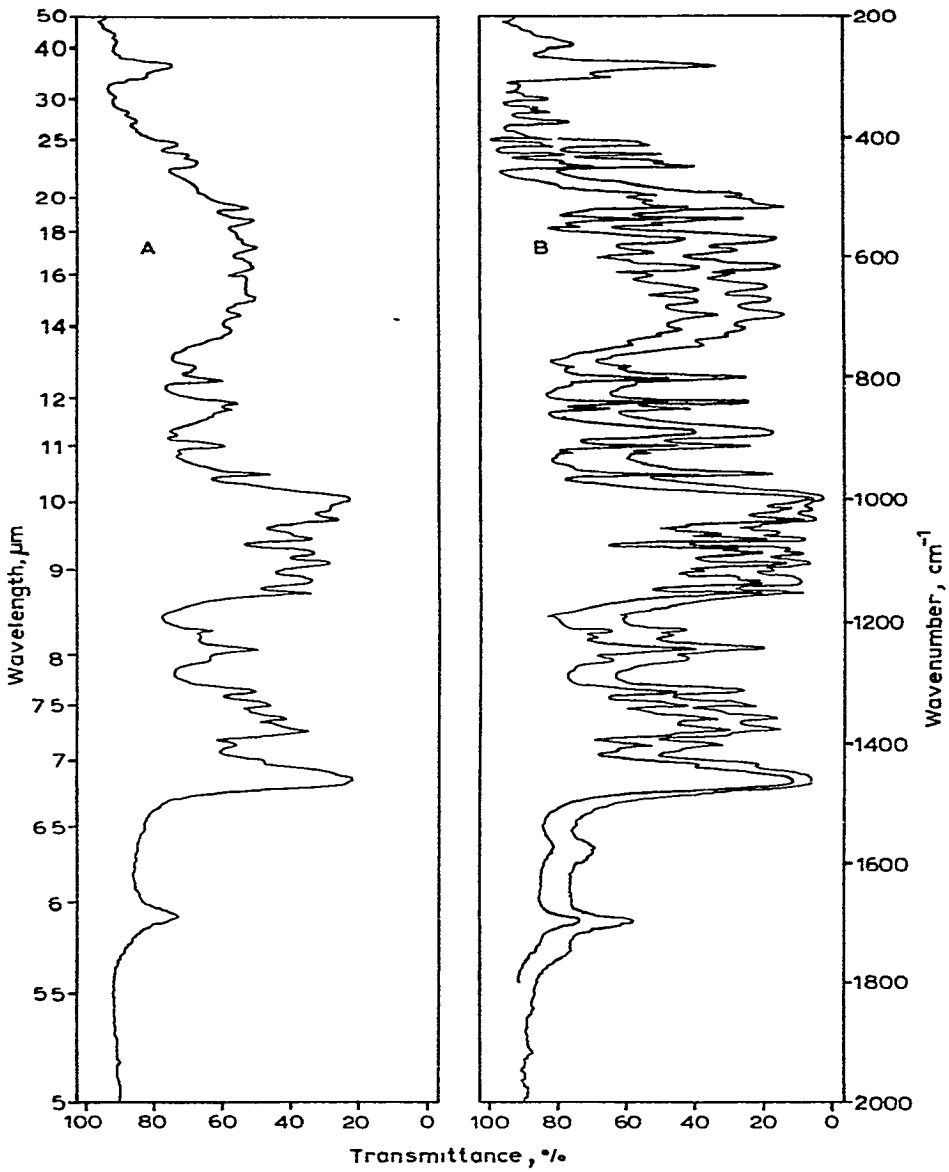


Fig 1 Part of the i r spectrum of a Nujol mull of α,α-trehalose dihydrate (A, at room temperature, B, at about 110 °K)

on which the bands are superimposed at 110 °K is almost completely removed at 20 °K

Parallel results are obtained with the OH stretching vibrations at higher frequencies Fig. 5 gives the spectrum of a mull of lactose monohydrate in this region at 298 °K, ~110 °K, and ~20 °K It may be seen that, on cooling, the bonded-OH band breaks up and shows much fine structure The monomer OH stretching-frequency appears at ~3525 cm^{-1} Apparently, one hydroxyl group of lactose is

not hydrogen-bonded in the crystal. This situation is similar to that found with sucrose, which is known to have one hydroxyl group unbonded[7]

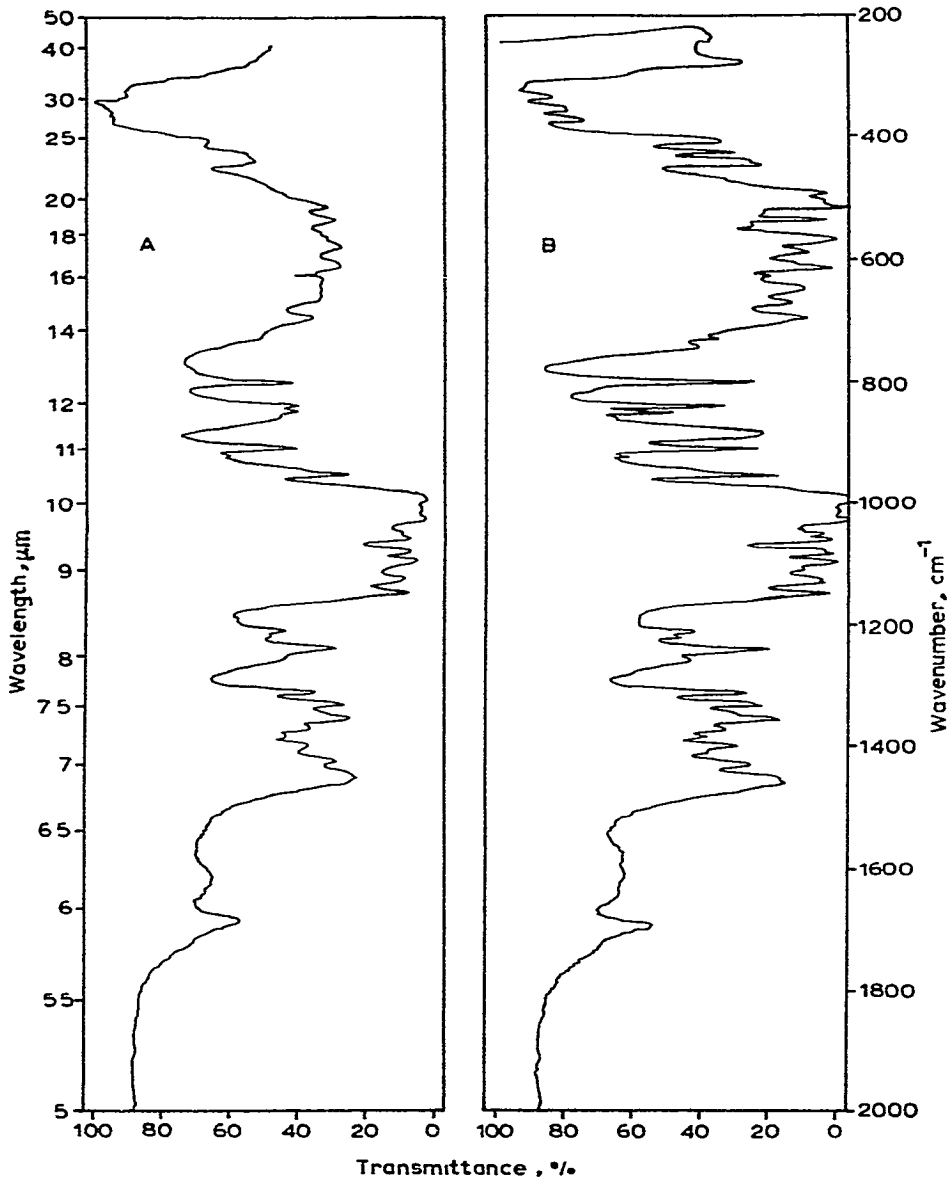


Fig 2 Part of the i r spectrum of α,α-trehalose dihydrate in a KBr pellet. (A, at room temperature, B, at about 110°K)

X-ray diffraction patterns — The powder X-ray diffraction patterns of a number of compounds were recorded in order to supplement or confirm information gained from i r. spectroscopy. The two most important factors were. (*I*) an estimate

of the percentage crystallinity of the compound, and (*2*) the presence or absence of phase changes between 298 and 110°K. In no compound studied was a gross change

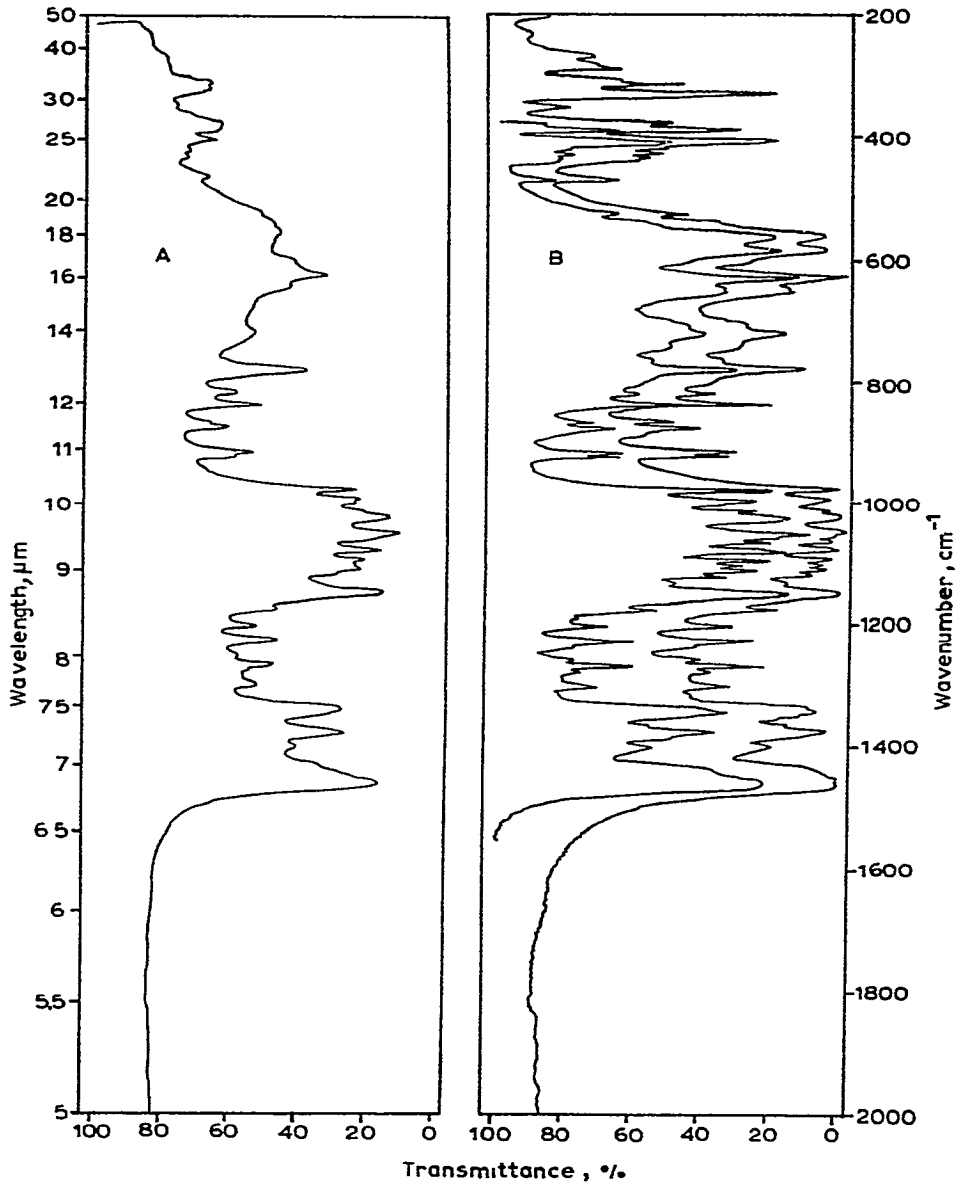


Fig 3. Part of the i r spectrum of a *ca* 1 1 mixture of D-glucose and D-fructose (A, at room temperature, B, at about 110°K.)

of phase detected by X-ray diffraction methods As the method is insensitive to positions of hydrogen atoms or to small changes within a molecule, there may be intramolecular disorder.

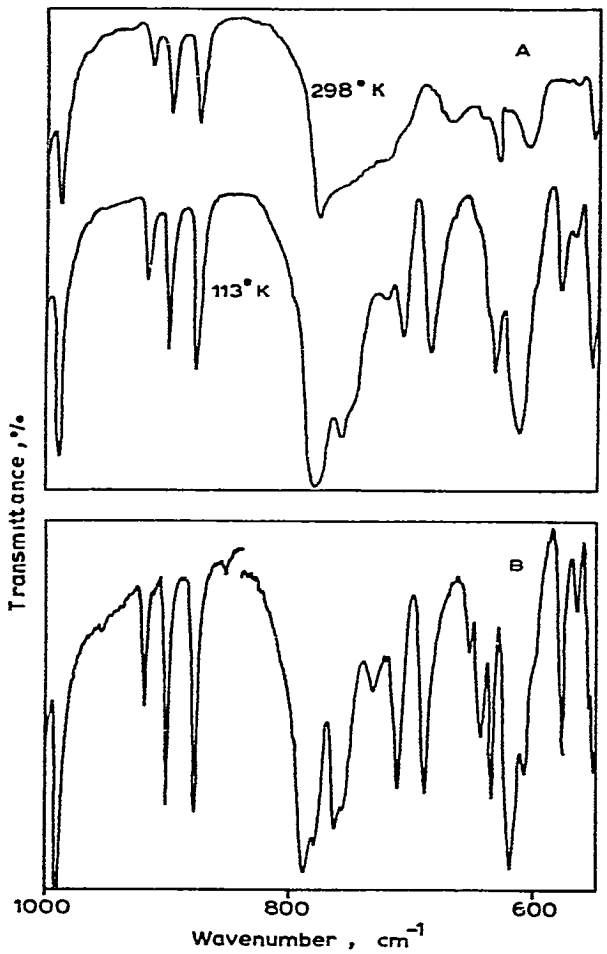


Fig 4 The ir spectrum of a Nujol mull of lactose monohydrate in the 1000–550 cm^{-1} region (A, at 298 °K and at 113 °K; B, at about 20 °K)

Although it is to be expected that lessening of thermal motion in the crystal should increase the intensity of the diffraction pattern at low temperature, this effect is fairly minor (5–10%) at the low temperatures used. Of somewhat more importance is the increase in intensity resulting from evacuation of the specimen-holding cell; this is caused by lessening of scattering by the air at atmospheric pressure, and an increase in intensity (of 10–15%) is obtained simply by evacuation

In some cases, a loss of intensity was observed on lowering the temperature; this occurred most often with hydrates or other materials that have a measurable vapor pressure at 110 °K. The prolonged period of evacuation necessary for complete scanning of the diffraction pattern results in vaporization of some of the material and, in the case of hydrates, can cause disruption of the lattice and a loss in crystallinity

The behavior of the X-ray diffraction pattern of urea with temperature has been reported[8], and this compound was used in checking the procedures, techniques, and apparatus Good agreement with the data reported was obtained.

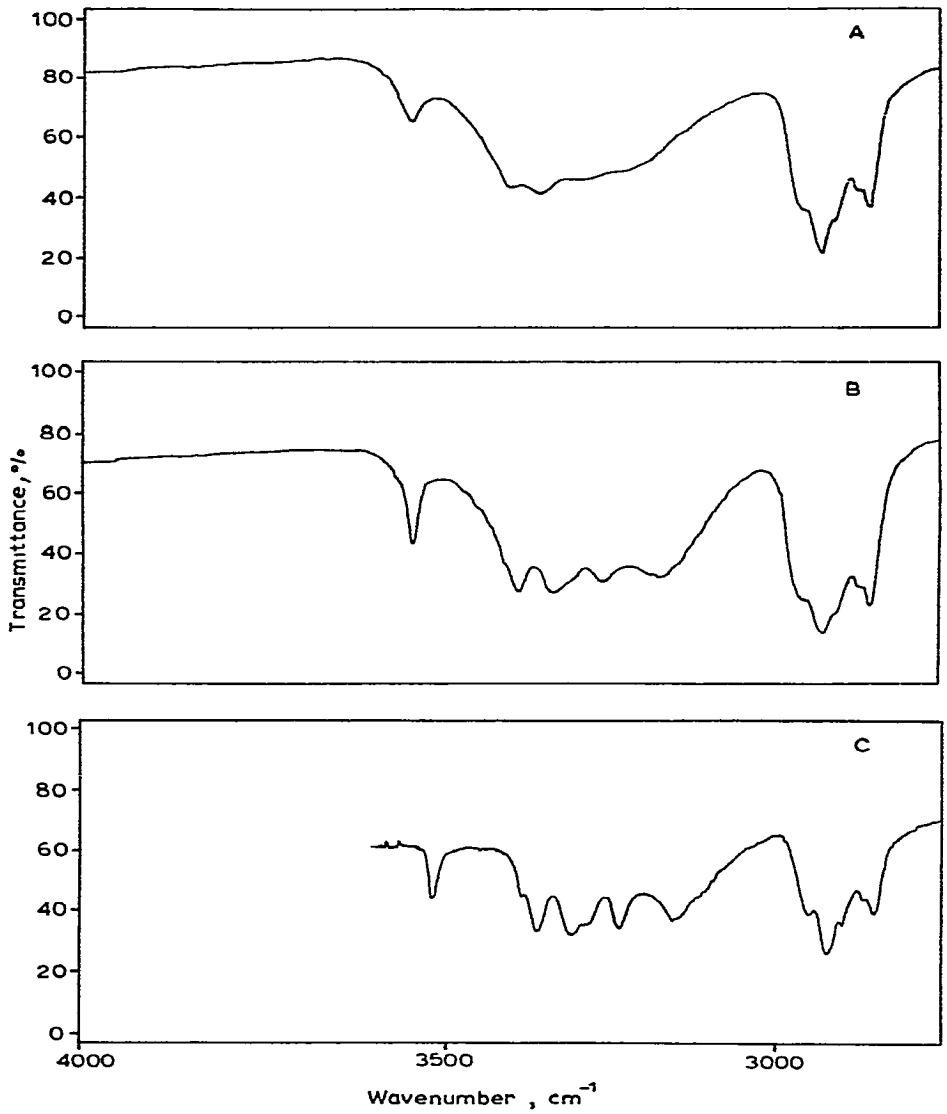


Fig 5. The i r spectrum of a Nujol mull of lactose monohydrate in the 3600–2800 cm^{-1} region (A, at room temperature, B, at about 110 °K, C, at about 20 °K)

In all compounds examined, except raffinose pentahydrate, there occurs, on cooling, a rather large contraction of the lattice that is not unexpected with organic molecules The reason for the lack of contraction of the lattice in raffinose pentahydrate is not yet known.

DISCUSSION

At present, a satisfactory explanation for the striking dependency of the i r spectra of these compounds on temperature is not available All of the compounds studied by Zhbankov *et al*[4] possess hydroxymethyl groups These workers noted changes, which they claimed to be sudden, in the spectra as the temperature was lowered. The changes were of two types (*a*) a shift of observed absorption bands to higher frequency, and (*b*) the appearance of new spectral features Although we have observed both of these types of change, our work indicates that the changes are not sudden, but are a continuous function of temperature Zhbankov *et al*[4] attributed their results to the formation of different crystalline structures that favored different rotamers in respect of the hydroxymethyl group, thereby giving rise to differences in the spectra This conclusion cannot be true, for two reasons. First, the X-ray diffraction data indicate that there is no change in the fundamental crystal-structure when the temperature is lowered (no phase change), and second, molecules that do not have hydroxymethyl groups, *e g*, D- or L-xylopyranose and D- or L-arabinopyranose, show the same effects quite clearly The shift of absorption bands to higher frequencies when the temperature is lowered is quite common, and has usually been ascribed to lattice contraction, although this can be only an approximation[9] The appearance of new spectral features can just as readily be explained by a very large band-sharpening and an increase in band intensity that "draws the bands out" from a diffuse, background absorption

However, there remains the possibility of internal rotation of the various hydroxyl groups Since this type of rotation changes the positions of hydrogen atoms only, the X-ray diffraction pattern would not reflect any change if the gross crystal structure is not disturbed on lowering the temperature Such an effect would result in a room-temperature crystal that would be quite highly ordered in terms of the heavy atoms, but that would possess a general disorder with respect to the hydrogen atoms bonded to oxygen atoms The changes in the spectra on lowering the temperature are, in some ways, similar to those observed with crystals known to have an order–disorder transition that is temperature-dependent, *e g*, ammonium chloride[10] An even closer analogy may be drawn with ice, whose major spectral features are well interpreted by a model involving long-range positional order in the crystal, but long-range orientational disorder[9,11]

Another effect that must certainly contribute to the results observed is the large diminution in intensity of difference bands and "hot" bands at low temperature. One of the major factors in the intensity of these bands is the frequency of the low-lying fundamental involved The lower this frequency, the more intense will these bands be at any given temperature Since complex compounds generally have a large number of fundamentals of very low frequency, many difference bands and "hot" bands are possible.

As crystalline solids are under consideration, these same mechanisms arise with the various optical and acoustical vibrations of the crystal. They are found at

very low frequencies, and their coupling with molecular vibrations again gives rise to difference bands. This kind of coupling has often been shown to occur, and its effect on the spectra can be striking[12] Of course, the "hot" bands of the optical lattice modes can also contribute to the observed spectrum These effects have also been observed, and discussed, in connection with the spectrum of ice[9]

Fig 6 shows the far-infrared spectrum of α,α-trehalose dihydrate at room temperature and near the temperature of boiling nitrogen The room-temperature spectrum shows that this compound does, indeed, have a large number of low-frequency absorption bands that would be expected to give rise to many difference bands and "hot" bands at higher frequencies The strong absorption band at ~67 cm^{-1} (at room temperature) is, apparently, a "hot" band; its intensity is about the same as that of the band at ~76 cm^{-1} On cooling the sample, both bands

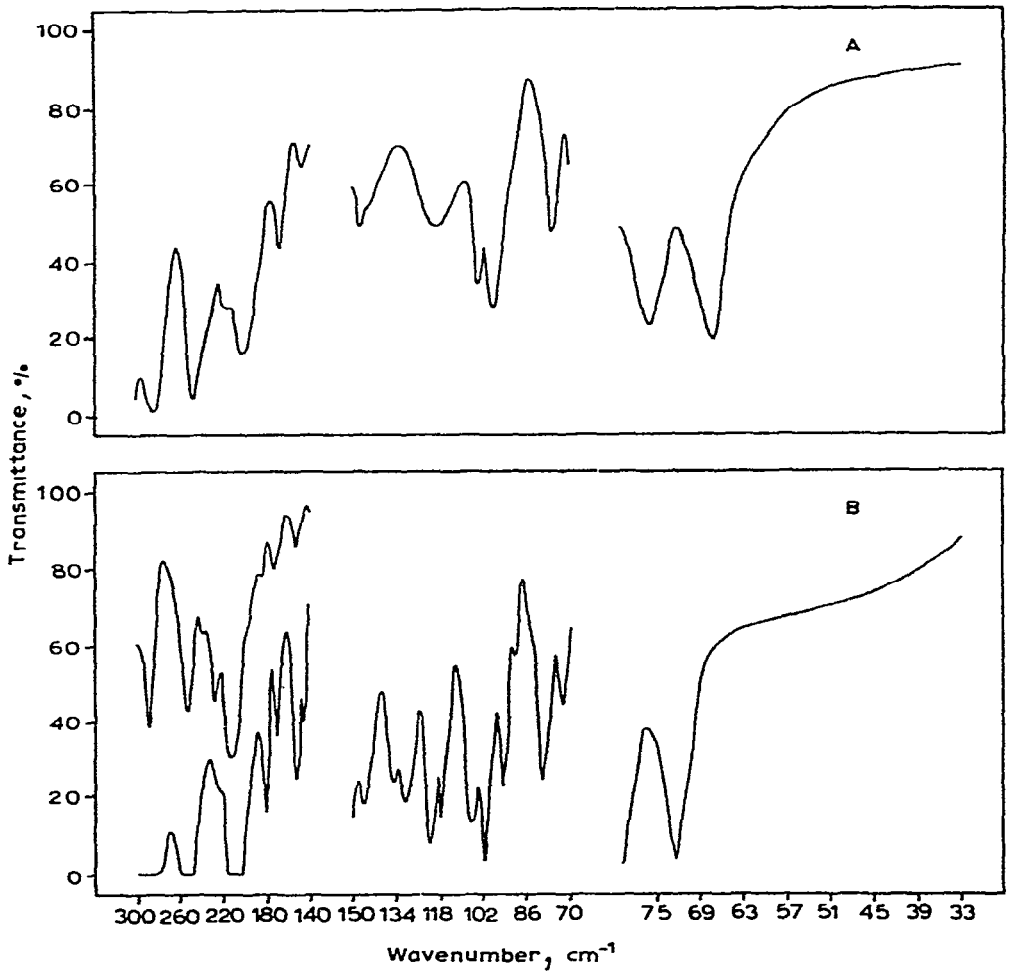


Fig 6. The far-infrared spectrum of a Nujol mull of α,α-trehalose dihydrate (A, at room temperature, B, at about 110 °K)

shift to higher frequency, but now the lower-frequency band is much less intense than the higher-frequency one; this is, of course, exactly what is expected of a "hot" band[13]. Again, the low temperature causes appearance, in the spectrum, of a great deal of fine structure not observed at the higher temperature

Although, for the phenomena observed, all of the possible reasons discussed are general in nature and would be expected to contribute to the final effect, further work is necessary in order that the observed results may be explained quantitatively To date, the striking results obtained on lowering the temperature have only been found with compounds that can undergo hydrogen-bonding Unfortunately, hydrogen bonding is not yet completely understood, and, as a result, it is difficult to determine those characteristics of the hydrogen bond that are important to this effect

ACKNOWLEDGMENTS

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2-CHLORO-2-DEOXY-D-GLUCOSE AND 2,2-DICHLORO-2-DEOXY-D-*arabino*-HEXOSE

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ABSTRACT

Chlorination of 3,4,6-tri-*O*-acetyl-D-glucal in daylight afforded 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-α-D-glucopyranosyl chloride (**2**) and 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranosyl chloride (**3**) in the ratio *ca* 4·1 Hydrolysis of compound **2** with hydrochloric acid gave 2-chloro-2-deoxy-D-glucose in high yield, thereby providing a much-improved route to this compound The mannosyl chloride **3** afforded 2-deoxy-D-*arabino*-D-hexose, with evolution of chlorine, on treatment with hydrochloric acid, and 3,4,6-tri-*O*-acetyl-D-glucal on treatment with sodium iodide in acetone

Dehydrohalogenation of the glucosyl chloride **2** with triethylamine afforded 3,4,6-tri-*O*-acetyl-2-chloro-1,2-dideoxy-D-*arabino*-hex-1-enopyranose, chlorination of which gave 3,4,6-tri-*O*-acetyl-2,2-dichloro-2-deoxy-D-*arabino*-hexosyl chloride Hydrolysis of the trichloro derivative with hydrochloric acid yielded crystalline 2,2-dichloro-2-deoxy-D-*arabino*-hexose

INTRODUCTION

Certain tumours apparently have more extensive glycolytic activity[1] than do the corresponding normal tissues, and considerable interest has been aroused in glycolysis inhibitors[2] and their effect on tumour growth Since the hexokinase stage appears[3] to be rate determining in the glycolytic sequence, we have begun a search for hexokinase inhibitors with the dual purpose of evaluating gross, anti-tumour activity *in vivo* and establishing structure–activity relationships from studies of isolated isozymes[4] of normal and tumour cells

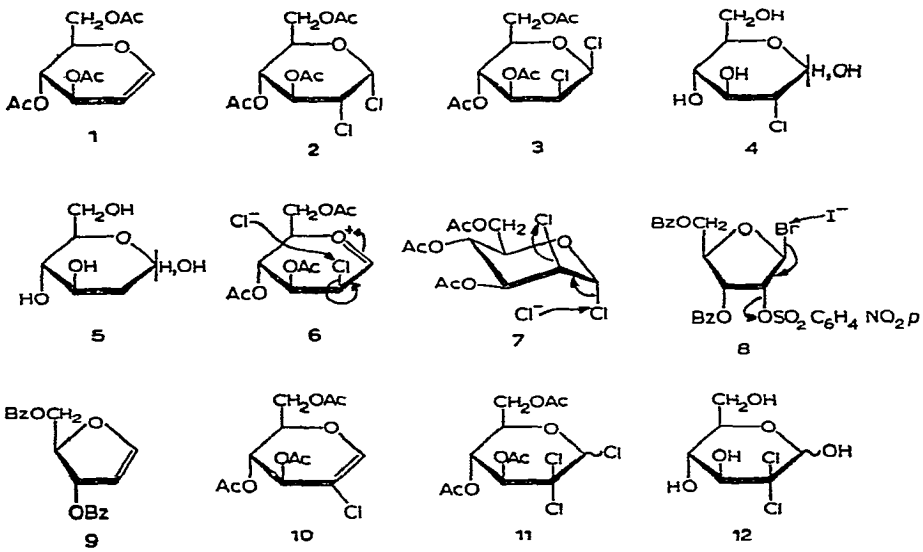
The affinity of derivatives of D-glucose for hexokinases (as indicated by K_m values) indicates[3] that positions 1, 3, 4, and 6 are importantly involved in the active site, since removal of the hydroxyl groups or inversion of configuration variously at these positions diminishes the affinity for the enzyme Position 2 is apparently exceptional, since D-mannose and 2-deoxy-D-*arabino*-hexose have low K_m values It is not known whether the hydroxyl groups at positions 1, 3, 4, and 6 in D-glucose are involved in proton donation or acceptance in hydrogen bonding within the enzyme–substrate complex, and it may be possible to segregate these

effects by replacing hydroxyl groups by chlorine or fluorine atoms, since they can function only as proton acceptors Both functions would be eliminated in deoxy sugars Additionally, the electronic effect of halogen atoms, especially fluorine, could affect the acidity of neighbouring hydroxyl groups and thereby the hydrogen-bonding characteristics and the formation of an enzyme–substrate complex In this connection, the 2,2-dihalogeno derivatives of D-glucose are of particular interest

Thus, there is a need for the synthesis of the complete series of chlorodeoxy and deoxyfluoro derivatives of D-glucose, of which only the following are presently known. 2-chloro-2-deoxy[5], 3-chloro-3-deoxy[6], 3-deoxy-3-fluoro[7], 6-chloro-6-deoxy[8], 6-deoxy-6-fluoro[9], the α-[10] and β-D-glucopyranosyl fluorides[11], and 2,2-dichloro-2-deoxy-D-*arabino*-hexosyl chloride[12]

RESULTS AND DISCUSSION

As part of a programme concerned with deoxyhalogeno derivatives of hexoses[7,13], we now report the synthesis of 2,2-dichloro-2-deoxy-D-*arabino*-hexose (**12**) by a route similar to that employed[14] for the conversion of 3,4-di-*O*-acetyl-D-arabinal into 2,2-dichloro-2-deoxy-D-*erythro*-pentose When this work was essentially complete, Bradley and Buncel[12] reported a similar synthetic approach which, however, proceeded only to the penultimate stage Since there was some overlap of work, only the results which differ significantly from those noted by Bradley and Buncel[12] and earlier investigators[15,16] are reported herein



1 2 3 4 5 6 7 8 9 10 11 12

Chlorination of 3,4,6-tri-*O*-acetyl-D-glucal (**1**) in carbon tetrachloride at <0° in daylight affords a mixture of 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-α-D-gluco-pyranosyl chloride (**2**) and 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranosyl

chloride (3) in the approximate ratio 4 1. Compounds 2 and 3, the configurations of which have been assigned by n m r. spectroscopy[12,16c], are formed by *cis*-addition of chlorine to the glucal derivative 1 The mechanism of the reaction has been considered in detail[16a c].

The isomeric glycosyl chlorides 2 and 3 were readily separable on a macroscale by column chromatography on silica gel, thereby providing an isolation procedure that is much more convenient than those previously reported[12,16b].

Hydrolysis of the glucosyl chloride 2 with boiling 0 1N hydrochloric acid gave 2-chloro-2-deoxy-D-glucose (4) in good yield The chloro sugar 4 is therefore much more readily available by this route than by the previously described method which involved conversion[12,15] of the 3,4,6-tri-*O*-acetyl-D-glucal–chlorine adduct into 1,3,4,6-tetra-*O*-acetyl-2-chloro-2-deoxy-D-glucose, followed by hydrolysis with hydrochloric acid

When the mannosyl chloride 3 was treated with boiling 0.1N hydrochloric acid, 2-deoxy-D-*arabino*-hexose (5, 2-deoxy-D-glucose) was formed in high yield, concomitant with the evolution of chlorine, no other product was detected It is improbable that a *cis*-elimination of chlorine occurs from the β-D-mannosyl chloride 3 Several other, more-reasonable possibilities exist For example, loss of chloride ion from the β-D-mannosyl chloride 3, before or after anomerisation (direct or by chloride exchange) into the α-D anomer, to give an oxocarbonium ion, followed by loss of chlorine, as shown in formula 6, would give 3,4,6-tri-*O*-acetyl-D-glucal (1) (or D-glucal if deacetylation occurs first) Normal, acid-catalysed hydration[17] then converts the glycol 1 into the 2-deoxy sugar 5 Alternatively, anomerization could be followed, as shown in formula 7, by attack of chloride ion at Cl-1 (or at Cl-2 with a similar consequence) with elimination of chlorine from the *trans*-diaxial dichloride and formation of 3,4,6-tri-*O*-acetyl-D-glucal (1) A closely related mechanism must be operative in the conversion[18] of 3,5-di-*O*-benzoyl-2-*O*-(*p*-nitrobenzenesulphonyl)-β-D-ribofuranosyl bromide (8) into 3,5-di-*O*-benzoyl-1,2-dideoxy-D-*erythro*-pent-1-enofuranose (9) on treatment with sodium iodide in acetone Iodide displacement, followed by an iodide ion-mediated elimination of the mechanistic type shown in formula 7, is a probable explanation for our observation of the formation of 3,4,6-tri-*O*-acetyl-D-glucal on treatment of the β-D-mannosyl chloride 3 with sodium iodide in acetone

Treatment of the glucosyl chloride 2 with triethylamine in boiling acetonitrile resulted in smooth dehydrochlorination (since H-2 and C^1-l are *trans*-diaxially disposed) to give 3,4,6-tri-*O*-acetyl-2-chloro-1,2-dideoxy-D-*arabino*-hex-1-enopyranose (10) The mannosyl chloride 3 was not affected under these basic conditions Chlorination of the chloroglycal 10 proceeded readily at 0° to give 3,4,6-tri-*O*-acetyl-2,2-dichloro-2-deoxy-D-*arabino*-hexosyl chloride (11) The α-D configuration has been assigned[12] to this compound by analogy with the structure of the major product (2) formed in the chlorination of 3,4,6-tri-*O*-acetyl-D-glucal (1) Hydrolysis of the trichloride 11 with boiling N hydrochloric acid gave the highly crystalline 2,2-dichloro-2-deoxy-D-*arabino*-hexose (12)

That the dichloro sugar **12** was essentially a pure anomer was indicated by the n.m r. spectrum in methyl sulphoxide (~10% solution, internal tetramethylsilane) which showed, *inter alia*, distinct 1-proton doublets for hydroxyl protons at τ 2 60, 4 00, and 4.80, together with a doublet for H-1 at 5 20 which was partly superimposed on a 1-proton triplet (HO-6) at 5 45 On addition of D_2O, the signals for hydroxyl protons disappeared, and the signal for H-1 collapsed into a singlet If, by analogy[19], the low-field signal (τ 2 60) is assigned to HO-1, the magnitude (~6Hz) of $J_{H\text{-}1,HO\text{-}1}$ is indicative[19] of an equatorial hydroxyl group, *i e*, the β-D configuration The n m r spectrum of compound **12** in deuterium oxide (~10% solution, internal *tert*-butyl alcohol) at room temperature contained (immediately after dissolution) two singlets at τ 4 60 and 4 95 (ratio *ca* 1 4) assignable to anomeric protons The ratio had changed to *ca* 1 3 after 72 h and was unchanged thereafter The $\Delta\tau$ value (0 35) for these signals is typical[20] for pyranoses, and, since the signal for an axial proton is usually[20] at higher field, the signals at 4 60 and 4 95 may be provisionally assigned to the α- and β-forms of 2,2-dichloro-2-deoxy-D-*arabino*-hexopyranose It also follows that the crystalline anomer **12** has the β-D configuration The mutarotation [$-3°$ (2 min)$\rightarrow+7°$ (2 h), water] of the crystalline anomer is also consistent with the β-D configuration

With acetic anhydride–pyridine, the dichloro sugar **12** gave a single tetraacetate to which the β-D configuration is tentatively assigned Acetylation of sugars with this reagent usually proceeds without a change in configuration at the anomeric centre

The biological properties of 2-chloro-2-deoxy-D-glucose (**4**) and 2,2-dichloro-2-deoxy-D-*arabino*-hexose (**12**) will be reported elsewhere, but it may be noted that the former compound partially inhibits the anaerobic yeast fermentation of D-glucose[21]

EXPERIMENTAL

General methods — Solutions were concentrated under diminished pressure with a rotary-type evaporator at temperatures below 50° Melting points were determined on a Kofler block apparatus and are corrected Optical rotations were measured with a Perkin–Elmer Automatic Polarimeter Type 141 Infrared spectra were obtained on a Perkin–Elmer 257 spectrophotometer N m r spectra were determined at 60 MHz with a Perkin–Elmer R10 spectrometer, using *ca* 15% solutions in $CDCl_3$, D_2O, or $(CD_3)_2SO$, tetramethylsilane or *tert*-butyl alcohol were used appropriately as internal standards Thin-layer chromatography (t l c) was carried out by the ascending method, using silica gel G (Merck, 7731) and detection with conc. sulphuric acid at 120°. Column chromatography was performed on silica gel G (Merck, 7734)

Chlorination of 3,4,6-tri-O-acetyl-D-glucal — 3,4,6-Tri-*O*-acetyl-D-glucal (13.6 g) was dissolved in carbon tetrachloride (100 ml) and cooled in an ice–salt mixture. Dry chlorine was passed through the solution until the chlorine colour persisted

(15 min); the reaction was carried out in normal daylight. The solution was stirred for 30 min, and the excess of chlorine was removed by aeration Examination of the solution by t.l c [light petroleum (b p 40–60°) –ether (4 1)] indicated two components with R_F values 0 6 and 0 4 Silica gel (30 g) was added to the solution which was then evaporated. The residue was divided and added to the tops of 2 columns of silica gel (250 g each) slurried with light petroleum, and elution was effected with light petroleum (b p 40–60°) –ether (2 1) to afford, first, 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-α-D-glucopyranosyl chloride (**2**, 12 6 g, 73%), m p 96–97° (from light petroleum–ether, 4 1), $[\alpha]_D^{25}$ +227 8° (*c* 1, chloroform), lit [16c], m p. 99–100°, $[\alpha]_D$ +227 6° (chloroform)

Eluted second was 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranosyl chloride (**3**, 4 g, 18%), m p 137–138° (from light petroleum–ether, 4 1), $[\alpha]_D^{25}$ −39 5° (*c* 0 23, chloroform), lit [16c], m p 145.5–146°, $[\alpha]_D^{25}$ −44° (chloroform)

2-Chloro-2-deoxy-D-glucose (**4**) — 3,4,6-Tri-*O*-acetyl-2-chloro-2-deoxy-α-D-glucopyranosyl chloride (**2**, 0 5 g) was refluxed with 0 1N hydrochloric acid (10 ml) until dissolution was complete (30 min) The solution was refluxed for a further 3 h, and was then cooled, neutralised with silver carbonate, filtered, and evaporated Elution of the residue from silica gel (25 g) with ethyl acetate–ethanol gave the title compound (0 26 g, 89%), m p 149°, $[\alpha]_D^{25}$ +56° (2 min) → +71° (equil , *c* 1.1, water), lit.[5], m p 147–148°, $[\alpha]_D^{25}$ +71 1° (water) (Found C, 36 3, H, 5 3, Cl, 18 2 $C_6H_{11}ClO_5$ calc · C, 36 3, H, 5 6; Cl, 17 9%)

Treatment of 2-chloro-2-deoxy-D-glucose (200 mg) with pyridine (2 ml) and acetic anhydride (1 ml) for 3 h at room temperature in the usual manner gave 1,3,4,6-tetra-*O*-acetyl-2-chloro-2-deoxy-β-D-glucopyranose (233 mg, 57%), m p. 108–110°, $[\alpha]_D^{25}$ +52 8° (*c* 1 0, chloroform); the product obtained[5] by treatment of the crude 3,4,6-tri-*O*-acetyl-D-glucal–chlorine adduct with silver acetate had m.p 111–112°, $[\alpha]_D^{25}$ +54 5° (chloroform) (Found C, 46 6, H, 5 2; Cl, 9 4 $C_{14}H_{19}ClO_9$ calc C, 46 9; H, 5.2, Cl, 9.7%)

The n m r spectrum of the tetra-acetate showed, *inter alia*, a doublet for H-1 at τ 4 2 ($J_{1\,2}$ 10 Hz) indicative of the β-D configuration at the anomeric centre

*Hydrolysis of 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-D-mannopyranosyl chloride* (**3**) — A solution of the title compound (1 5 g) in 0 1N hydrochloric acid (25 ml) was refluxed until dissolution was complete and then for a further 8 h Examination of the hydrolysate by t l c revealed a single product The evolution of chlorine from the solution, detected by odour, was confirmed by the liberation of iodine from starch–iodine The solution was neutralised with silver carbonate, filtered, and evaporated Elution of the resulting, yellow syrup from silica gel with ethyl acetate gave 2-deoxy-D-*arabino*-hexose (**5**, 2-deoxy-D-glucose) (0 68 g, 79%), as needles, m p 148° (from ethyl acetate–ethanol), $[\alpha]_D^{25}$ +41.5° (5 min)→ +47 2° (12 h, equil., *c* 1 0, water), lit [22], m p 146°, $[\alpha]_D$ +38 3°→ +45 9° (water)

*3,4,6-Tri-*O*-acetyl-2-chloro-1,2-dideoxy-D-arabino-hex-1-enopyranose* (**10**). — A solution of 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-α-D-glucopyranosyl chloride (**2**, 20 g) in acetonitrile (100 ml) and triethylamine (20 ml) was refluxed for 72 h It was difficult

to monitor the reaction by t l c. because of the similarity in R_F values of the starting material and product The solution was cooled, silica gel (30 g) was added, and the solvent was evaporated The dry residue was divided and added to the tops of 2 columns of silica gel (250 g each) dispersed in light petroleum; elution with light petroleum–ether (b.p. 40–60°) (4:1) afforded the crude chloroenol ether (15 5 g, 87%) as a pale-yellow oil, which on distillation gave the title compound, b p. 132°/0 025 mm, $[\alpha]_D$ +19° (*c* 1, chloroform) (Found· C, 47 2; H, 5 0. $C_{12}H_{15}ClO_7$ calc . C, 47 0; H, 4 9%) The i r. spectrum (liquid film) contained a band at 1640 cm^{-1} (C=C). The n m.r. spectrum showed the following signals τ 3 27, singlet (H-1), 4 51, doublet ($J_{3\,4}$, 4 Hz, H-3); 4 76, triplet ($J_{3,4} = J_{4,5}$ 4 Hz, H-4), with fine splitting (~1 Hz) presumably due to coupling with H-6 and/or H-6′, 5 70, broad multiplet (H-5, H-6); 7 92, singlet (acetyl protons)

*Action of sodium iodide on 3,4,6-tri-*O*-acetyl-2-chloro-2-deoxy-β-*D*-manno-pyranosyl chloride* (3) — A solution of the title compound (1 g) in acetone (10 ml) was treated with a solution of sodium iodide (1 g) in acetone (10 ml) at room temperature. The iodine liberated was destroyed by the addition of sodium thiosulphate (2 g). The solution was refluxed for 12 h, and then cooled, filtered, and evaporated The syrupy residue was eluted from silica gel (25 g) with light petroleum (b p 40–60°) –ether (4·1 and 2.1) to afford 3,4,6-tri-*O*-acetyl-D-glucal (**1**, 0 23 g) (characterised by t l c and by n m r. and i r spectroscopy), m p 50–53°, together with starting material (0 4 g, 40% recovery)

*2,2-Dichloro-2-deoxy-β-*D*-arabino-hexose* (**12**) — 3,4,6-Tri-*O*-acetyl-2,2-dichloro-2-deoxy-α-D-*arabino*-hexopyranosyl chloride {2 g, m p. 162–163°, $[\alpha]_D$ −6° (*c* 2, chloroform)}, prepared by essentially the method of Bradley and Buncel[12], was refluxed in N hydrochloric acid (10 ml) until dissolution was complete (3 h) The solution was refluxed for a further 5 h, and then cooled, neutralised with silver carbonate, filtered, and evaporated to dryness. Recrystallisation of the residue from ethyl acetate–ethanol (4 1) gave the title compound as needles (1 15 g, 93%), m p 213–214°, $[\alpha]_D^{25}$ −3° (2 min)→+7° (2 h, equil , *c* 1 0, water), −6°→ +1 6° (21 days, still mutarotating) (*c* 1 3, methyl sulphoxide) (Found C, 31.2, H, 4 5, Cl, 29 9 $C_6H_{10}Cl_2O_5$ calc : C, 30 9; H, 4 3; Cl, 30 4%) Repetition of the hydrolysis, but with omission of the neutralisation stage, gave a similar result.

With acetic anhydride–pyridine under the usual conditions, the dichloro sugar **12** gave a tetra-acetate (84%), m p. 96–97°, $[\alpha]_D$ +10° (*c* 1 04, chloroform) (Found: C, 42 4; H, 4 6; Cl, 17 5. $C_{14}H_{18}Cl_2O_9$ calc . C, 41.9, H, 4 5, Cl, 17.7%) The n m r spectra ($CDCl_3$) of the crude acetate and recrystallised material were closely similar and contained the following signals· τ 4 00, singlet, H-1; 4 40, doublet ($J_{3\,4}$ ~9 Hz), H-3, 4 74, triplet ($J_{4\,5}$ ~9 Hz), H-4; 5 70–6 20, complex multiplet, H-5, H-6; 7 74, 7.80, 7.90, 7 93, singlets, acetyl protons

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STRUCTURAL STUDY OF POLYSACCHARIDES FROM THE OAK LICHEN *Evernia prunastri* (L.) ACH.

PART I. AN ALKALI-SOLUBLE GALACTOMANNAN*

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ABSTRACT

An acidic galactomannan, $[\alpha]_D^{20}$ +79° (water), containing D-galactose (36%), D-mannose (54%), and D-galacturonic acid (10%) has been extracted with dilute alkali from the oak lichen *Evernia prunastri* (L) Ach Hydrolysis of the fully methylated galactomannan yielded 2,3,4,6-tetra-*O*-methyl-D-galactopyranose (3 2 mol), 2,3,4,6-tetra-*O*-methyl-D-mannopyranose (1 mol), 3,4,6-tri-*O*-methyl-D-mannopyranose (1.1 mol.), 3,4-di-*O*-methyl-D-mannopyranose (1 1 mol), 3,5-di-*O*-methyl-D-mannofuranose (0.9 mol), 4-*O*-methyl-D-mannopyranose (1 2 mol.), and 2,3,4-tri-*O*-methyl-D-galacturonic acid (1 mol) Since the results of periodate oxidation and Smith degradation were in agreement with methylation studies, it was concluded that the heteroglycan is a highly branched, acidic galactomannan, containing a basic chain of (1→2)- and (1→6)-linked mannose residues. The galactopyranose and galacturonic acid residues are present exclusively as non-reducing, terminal groups. On the basis of i r spectral data and enzymic hydrolysis, the galactopyranose residues are assigned the β-D configuration, whereas the mannose residues appear to be predominantly of the α-D type

INTRODUCTION

The carbohydrate part of the oak lichen *Evernia prunastri* (L) Ach. has not been extensively investigated Although the polysaccharides of this lichen were investigated[1] as early as 1864, no serious study was made until recently Muller[2] analysed the water-soluble polysaccharide called evernin, previously identified[1] as a glucan, and proposed its empirical formula Ullander and Tollens[3] wrongly concluded that galactose, in addition to glucose, was present in evernin Drake[4] subsequently described the isolation and some properties of evernin, and a more-detailed study was recently performed[5]. The alkali-soluble polysaccharides have not hitherto been investigated.

*Preliminary results were presented at the 4th International Symposium on the Chemistry of Natural Products (Stockholm, June 1966)

The present paper describes the structural study of an alkali-soluble heteroglycan isolated from the oak lichen *Evernia prunastri* (L) Ach

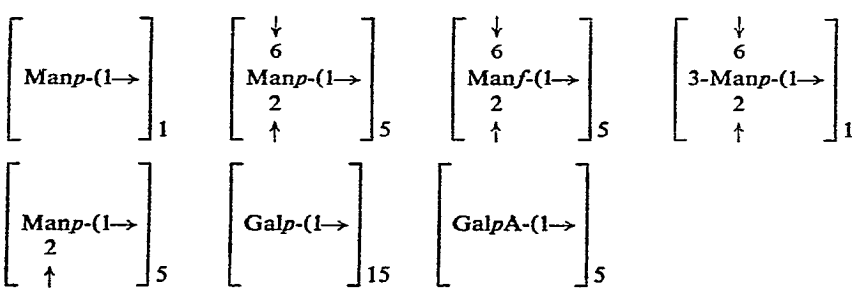
RESULTS AND DISCUSSION

The crude polysaccharide was obtained by extraction with 4% sodium hydroxide in a stream of nitrogen. Prior to the alkaline extraction, the lichen (collected from the Bosnian forests in the period of 1958–1959) was treated with methanol, followed by water, in order to remove soluble components Fractionation of the crude polysaccharide was effected by precipitation with barium hydroxide[6], Fehling's solution[7], and iodine[8].

The pure galactomannan was a cream-coloured powder, having $[\alpha]_D^{20}$ +79° (water), which was soluble in water and reduced Fehling's solution Acid hydrolysis followed by paper chromatography revealed galactose, mannose, and galacturonic acid Mannose and galactose were separated by preparative, paper chromatography and identified as crystalline derivatives The identity of the galacturonic acid was confirmed by reduction of the methyl ester to galactose Quantitative determinations by the phenol–sulphuric acid method[9] showed the presence of 37% of galactose, 50% of mannose, and 13% of galacturonic acid in the hydrolysate. The number-average degree of polymerization was found to be 45 ±1 units On acid hydrolysis, the specific rotation changed from +120 to +60°, indicating the predominance of α-D-glycosidic linkages This was confirmed by the infrared spectrum of the polysaccharide which contained bands at 840 and 902 cm^{-1} characteristic[10] of α-D linkages and probably of β-D galactopyranose residues The latter inference was confirmed on hydrolysis with β-D-galactosidase

The fully methylated galactomannan, which had $[\alpha]_D^{20}$ +47° (chloroform), was hydrolyzed with sulphuric acid to afford the mixture of methyl ethers shown in Table I This mixture was resolved by preparative thin-layer and paper chromatography, and the components were characterized by the formation of crystalline derivatives The quantitative results are given in Table I

The methylation results show that the polysaccharide contains the following structural units in the approximate ratios shown



The isolation of 2,3,4,6-tetra-*O*-methyl-D-galactose and 2,3,4-tri-*O*-methyl-D-galacturonic acid as the sole derivatives of galactose and galacturonic acid indicates

TABLE I

HYDROLYSIS PRODUCTS FROM METHYLATED GALACTOMANNAN FROM THE OAK LICHEN *Evernia prunastri* (L) ACH

	O-Methylglycoses	*Yield (wt %)*	*Molar proportion*
1	2,3,4,6-Tetra-*O*-methyl-D-mannopyranose	11	1
2	2,3,4,6-Tetra-*O*-methyl-D-galactopyranose	36	3 2
3	3,4,6-Tri-*O*-methyl-D-mannopyranose	12	1 1
4	3,5-Di-*O*-methyl-D-mannofuranose	8 3	0 9
5	3,4-Di-*O*-methyl-D-mannopyranose	11	1 1
6	4-*O*-Methyl-D-mannopyranose	11	1 2
7	2,3,4-Tri-*O*-methyl-D-galacturonic acid	10	1

that all of the residues of these sugars are present as terminal, non-reducing groups attached to the mannose backbone The nature of the isolated, methylated derivatives of mannose (**3**, **4**, **5**, and **6**) does not indicate an unambiguous structure. It is clear, however, that the unbranched portion of the galactomannan is formed by (1→2)-linked D-mannopyranose residues which give rise to 3,4,6-tri-*O*-methyl-D-mannopyranose on hydrolysis of the methylated polysaccharide The formation of 3,4-di-*O*-methyl-D-mannopyranose and 3,5-di-*O*-methyl-D-mannofuranose does not confirm whether the branching occurs at positions 2 or 6 of the mannose residues, or at both positions 4-*O*-Methyl-D-mannopyranose is derived from branch points involving D-mannopyranose residues substituted at positions 1, 2, 3, and 6

On the basis of the methylation data, it may be concluded that the galactomannan is a highly branched, acidic heteroglycan having a basic chain composed of mannopyranose and mannofuranose residues linked (1→2) and/or (1→6) The terminal, non-reducing groups are galactopyranose, galacturonic acid, and some mannopyranose residues The presence of mannofuranose and galacturonic acid residues makes this polysaccharide unique amongst those from lichens

The periodate-oxidation results were in accordance with those of the methylation study The galactomannan consumed 1 28 mol of reagent with concomitant liberation of 0.42 mol of formic acid (Table II), which is in good agreement with the calculated amounts (1.33 and 0 55 mol respectively) The oxopolysaccharide was further reduced and hydrolyzed to give glycerol, mannose, and traces of uronic acid

Partial, acid hydrolysis of the polysaccharide with 0 33N sulphuric acid gave a mixture of seven components The graph obtained by plotting[11] the logarithm of the partition functions (α') against the degree of polymerization of the oligosaccharides (Fig 1) indicates the presence of two homologeous series of oligosaccharides This result is consistent with the presence of two types of linkage in the basic chain

The simplest structure that can be proposed for the galactomannan from the oak lichen *E. prunastri* (L) Ach , based on the results of methylation and periodate oxidation, is shown in Fig 2

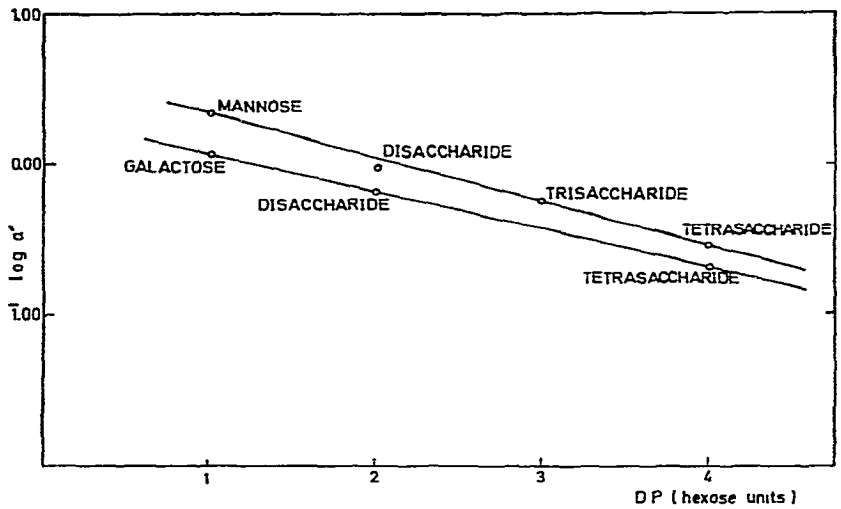


Fig 1 Graph of the logarithm of the partition function[11] (α') against D P of the oligosaccharides

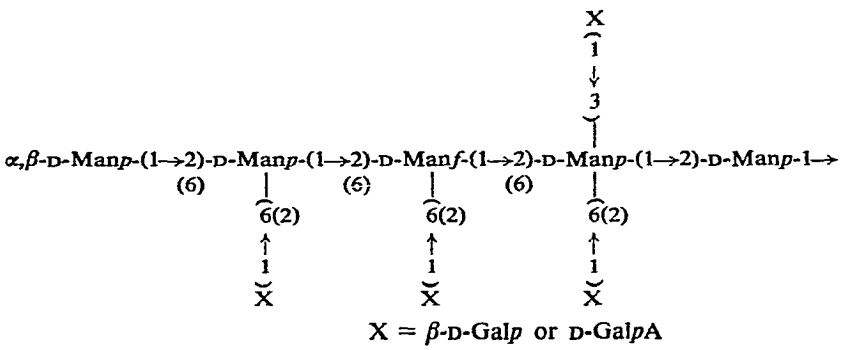


Fig 2 Proposed structure for the galactomannan from *E prunastri*

EXPERIMENTAL

Paper chromatograms were run by the descending method on Whatman No. 1 paper with the following solvent systems (v/v) (*A*) ethyl acetate–pyridine–water (8 2.1), (*B*) butyl alcohol–ethanol–water (5·1 4), (*C*) butyl alcohol–ethanol–water–ammonia (40.10 49.1), (*D*) butanone saturated with water, (*E*) butyl alcohol–ethanol–water (3·1 1), (*F*) ethyl acetate–acetic acid–water (3·1 3), and (*G*) ethyl acetate–pyridine–water (5 2 5) Whatman 3MM papers were used for preparative, paper chromatography. Thin-layer chromatography was performed on silica gel H (Merck) plates (13 × 20 × 0 5) with the solvents· (*H*) benzene–acetone (1 1), (*I*) butanone–water–ammonia (100 50 3), (*J*) butyl alcohol–acetic acid–ether–water (9.6 3 1), and (*K*) ethyl acetate–formic acid–water (3.1:3) Sugar components on paper chromatograms were detected with silver nitrate–sodium hydroxide[12], aniline hydrogen

phthalate[13], *p*-anisidine hydrochloride[14], or triphenyltetrazolium chloride[15] Spraying reagents for t l c were· aniline hydrogen phthalate, *p*-anisidine hydrochloride, or 50% sulphuric acid

All evaporations were performed at 35–40° under diminished pressure. Melting points are not corrected. Specific rotations are equilibrium values R_G values represent movement relative to that of 2,3,4,6-tetra-*O*-methyl-D-glucose

Isolation of the crude polysaccharides — Finely powdered, oak lichen (500 g) was extracted (Soxhlet) with ether (3 × 2-l) and with methanol (3 × 2-l) The air-dried residue was further extracted with distilled water (*ca* 10 litres) at 50° until the anthrone reaction was negative Extraction with 5% sodium carbonate solution was then performed in order to remove all acidic components The residual material was then extracted with 4% aqueous sodium hydroxide in a stream of nitrogen The mixture of polysaccharides, precipitated from the extract (6 l) by the addition of ethanol (8 l) and glacial acetic acid (1 6 l), was obtained as a light, cream-coloured powder (7% on the basis of dry lichen)

The crude polysaccharide was redissolved in 4% aqueous sodium hydroxide, the alkali was neutralized with glacial acetic acid (to pH 5), and the solution was dialyzed for 3 days against running tap-water and for 1 day against distilled water The dialyzate, after concentration *in vacuo* to a volume of 100 ml, was added to the same volume of ethanol The precipitate was separated by centrifugation and dried by solvent exchange with ethanol and ether A sample (5 mg) of this material was hydrolyzed with 2N sulphuric acid (0 5 ml) for 12 h at 95° in a sealed tube Paper chromatography (solvent *A*) revealed the presence of glucose, mannose, galactose, xylose, and a uronic acid

Isolation and purification of the galactomannan — The crude, polysaccharide preparation (10 g) was dissolved in 4% aqueous sodium hydroxide (500 ml, stream of nitrogen), and the solution was diluted with water (500 ml) To the stirred mixture, saturated, aqueous barium hydroxide was added until precipitation was complete The precipitate was remove by centrifugation and dissolved in 2N acetic acid, and the solution was dialyzed against running tap-water (2 days) and distilled water (1 day). After concentration to 500 ml, the dialyzed solution was added to 500 ml of ethanol The precipitated polysaccharide (5 g, 50% based on the crude polysaccharide) was separated by centrifugation and dried by solvent exchange Since paper chromatography of the hydrolyzed polysaccharide (solvent *A*) revealed galactose, mannose, a uronic acid, and traces of glucose, further purification was effected with Fehling's solution[7], and then four times by the iodine method[8] in order to remove traces of admixed glucan The resulting polysaccharide was a cream-coloured powder (ash, 0 2%, N, nil), having $[\alpha]_D^{20}$ +79 ±1° (*c* 1 13 water), which reduced Fehling's solution, and, on acid hydrolysis, gave galactose, mannose, and galacturonic acid (paper chromatography in solvents *A*, *C*, and *F*, t l c. in solvents *J* and *K*) The number-average degree of polymerization was 45 ±1 units, based on an estimation of the reducing end-group by hypoiodite oxidation[16]. The polysaccharide showed ν_{max}^{KB} 3450, 840, and 902 cm^{-1}.

Estimation and identification of constituent sugars. — The polysaccharide (36 mg) was hydrolyzed with 2N sulphuric acid in a sealed tube for 15 h at 95° After neutralization with barium carbonate (to pH 5), the solution was concentrated *in vacuo* and chromatographed in solvent *A* Neutral sugars were estimated by the phenol–sulphuric acid method[9] which indicated 37% of galactose, 50% of mannose, and 13% of galacturonic acid (calculated by difference) The amounts of *O*-methyl derivatives (Table I) in the hydrolyzate of the methylated polysaccharide corresponded to 36% of galactose, 54% of mannose, and 10% of galacturonic acid.

Another portion of hydrolyzate (100 mg) was subjected to preparative, paper chromatography (solvent *A*) to give D-mannose, characterized as the phenylhydrazone[17], m p and mixed m.p 199°, $[\alpha]_D^{20}$ +32° (*c* 1, pyridine), and D-galactose, m p 164–165°, $[\alpha]_D^{20}$ +80° (*c* 1 8, water), characterized by conversion into the phenylhydrazone[18], m p and mixed m p. 160°, $[\alpha]_D^{20}$ −21° (*c* 4, water)

The third component of the hydrolyzate was identified as galacturonic acid on the basis of its chromatographic mobility (R_F 0 13, paper, solvent *A*; and 0 33, t l c , solvent *K*) In a separate experiment, the galactomannan (109 4 mg) was hydrolyzed with N sulphuric acid for 15 h The acid was neutralized (barium hydroxide and barium carbonate), and the solution was deionized by Amberlite IR-120 (H^+) resin The acidic component was then adsorbed on Dowex-1 X8 (OH^-) resin, and neutral sugars were eluted with water (until the anthrone test was negative) The uronic acid was eluted with N sodium hydroxide and converted into the free acid by treatment with Amberlite IR-120 (H^+) resin The identity of the galacturonic acid was then established by the reduction of its methyl ester methyl glycoside with lithium aluminium hydride, followed by acidic hydrolysis, to afford galactose

Periodate oxidation — The galactomannan (244 4 mg) was dissolved in water (100 ml), and 0 1N sodium periodate (100 ml) was added Oxidation was allowed to proceed at 20° in the dark Aliquots were removed at intervals for an estimation of periodate and formic acid[19], the results are given in Table II The data were extrapolated to zero time to give net values of 1 28 mol of periodate consumed and 0 42 mol of formic acid released per "anhydrohexose" unit.

TABLE II

PERIODATE OXIDATION OF THE GALACTOMANNAN

Time (h)	20	44	68	92	116
Periodate[a]	1 22	1 39	1 44	1 50	1 50
Formic acid[a]	0 31	0 36	0 41	0 42	0 42

[a]In moles per mole of "anhydrohexose"

After the oxidation was complete, 0 5M barium acetate was added to precipitate periodate and iodate The filtered solution, after deionization [Amberlite IR-120 (H^+) and IR-45 (OH^-) resins], was concentrated to a small volume (50 ml) and then treated with potassium borohydride[20] (200 mg) After 48 h, the solution was neutral-

ized with acetic acid, deionized, and evaporated to dryness The last traces of boric acid were removed from the residue by repeated evaporation with methanol The residue (125 mg) was then hydrolyzed with 0 1N sulphuric acid for 15 h at 95° Paper chromatography revealed the presence of glycerol (R_F 0 39, solvent *E*) and mannose (R_F 0 18, solvent *E*), in addition to a slow-moving, unidentified component (R_F 0 12) T l c (solvent *J*) proved the presence of glycerol (R_F 0 58) and mannose (R_F 0 55)

Methylation of the galactomannan — The galactomannan (3 g) was methylated in the usual way, first by the Haworth procedure (three times) and then five times by treatment with silver oxide and methyl iodide, to yield the fully methylated galactomannan (1 9 g), $[\alpha]_D^{20}$ +47 ±1° (*c* 1 1, chloroform), which did not show hydroxyl absorption in the infrared (Found OCH_3, 44 2%)

Hydrolysis of the methylated galactomannan — The methylated polysaccharide (1 5 g) was dissolved in ice-cold 72% sulphuric acid (7 ml), and the solution was kept for 2 h at room temperature Water (49 ml) was then cautiously added to the hydrolyzate (previously cooled in ice), and the diluted solution was then heated for 4 h on a boiling water-bath The hydrolysate was diluted with water (50 ml), neutralized (barium carbonate), filtered, and evaporated *in vacuo* below 40° The residue was then successively extracted with methanol and chloroform, and the combined extracts were evaporated Paper chromatography in solvents *B*, *C*, and *D* revealed the presence in the residue of five components, whereas t l c in solvents *H* and *I* showed the presence of at least six components

The mixture of methylated sugars was first resolved by preparative t l c in solvent *H*, using double development. Fractions that were not homogeneous were resolved by preparative, paper chromatography in solvents *B* and *E* The following fractions were obtained

Fraction 1 The syrup (6 6 mg) was chromatographically homogeneous with a mobility corresponding to that of 2,3,4,6-tetra-*O*-methyl-D-mannose[21] [R_G 0 96 (solvent *B*) and 0 98 (solvent *D*)] The crystals which separated on storage had m p. and mixed m p 50° (from light petroleum), $[\alpha]_D^{22}$ +11→+2° (*c* 0 5, water) The aniline derivative[22] had m p and mixed m p 145° (from ether–methanol 98 2) $[\alpha]_D^{22}$ −11 ±1° (*c* 0.8, methanol)

Fraction 2 (19 5 mg) was syrupy 2,3,4,6-tetra-*O*-methyl-D-galactose[23] [R_G 0 88 (solvent *B*) and 0 68 (solvent *C*)], $[\alpha]_D^{20}$ +118 ±1° (*c* 1 9, water) The aniline derivative[22] had m p and mixed m p 191–193° (from ethyl acetate), $[\alpha]_D^{20}$ +37 ±1° (*c* 1, acetone)

Fraction 3 (6 5 mg) was 3,4,6-tri-*O*-methyl-D-mannose[24], m p and mixed m p 104°, R_G 0 79 (solvent *B*), $[\alpha]_D^{21}$ +36 ±1° (*c* 0 6, methanol) D-methylation with hydrogen bromide gave mannose as the major product Oxidation with bromine yielded a lactone having physical constants, m p 96–97° (from ether), $[\alpha]_D^{20}$ +160→+111° (*c* 0 5, water), in good agreement with those reported for 3,4,6-tri-*O*-methyl-D-mannonolactone[24]

Fraction 4 (9 8 mg) contained two components [R_G 0 31 and 0 35 (solvent *C*)

and 0.67 and 0 72 (solvent *D*), corresponding to di-*O*-methyl sugars Demethylation with hydrogen bromide gave only mannose. T l c. (solvent *I*) confirmed that fraction 4 was a mixture of two components (R_F 0.82 and 0.78), and a portion (64 mg) of the hydrolysate was resolved by preparative, paper chromatography (solvent *E*) to give fraction 4A (5 4 mg), R_G 0 67 (solvent *E*), which crystallized to give material having physical constants, m p 125–126° (from butanone), $[\alpha]_D^{20}$ +14 ±1° (*c* 1, water), corresponding to those for 3,5-di-*O*-methyl-D-mannose[25], and fraction 4B (7 1 mg) which was 3,4-di-*O*-methyl-D-mannose monohydrate[26], m p and mixed m p 71–73°, $[\alpha]_D^{21}$ +32 ±1° (*c* 0 6, methanol)

Fraction 5 (11 4 mg), which contained two components (paper chromatography in solvents *B* and *C*), was resolved (solvent *B*) into fractions 5A and 5B. Fraction 5A (5 3 mg), R_G 0 32 (solvent *B*), R_F 0 13 (solvent *C*), $[\alpha]_D^{20}$ +32→+22° (*c* 0 5, water), had m p 130° (from absolute methanol) These values correspond closely to those for 4-*O*-methyl-D-mannose[27], the lack of material precluded the formation of a derivative Fraction 5B (4 8 mg), R_G 0 25 (solvent *B*), was reduced with lithium aluminium hydride in the usual way. The product obtained had the same chromatographic mobility (solvents *B* and *D*) as 2,3,4-tri-*O*-methyl-D-galactose[28] Crystallization from methanol–water (9.1) yielded product melting at 101°, $[\alpha]_D^{20}$ +126→+106° (*c* 0 7, water)

Enzymic hydrolysis of the galactomannan — The galactomannan (500 mg) was dissolved in water and incubated in dialysis tubing[29] with yeast β-galactosidase (50 mg in 10 ml of 0 02M phosphate buffer, pH 7) for 4 days at 37° A few drops of toluene were added to prevent microbial contamination Analysis of the concentrated dialysate by paper chromatography revealed galactose as the only component

Partial, acid hydrolysis of the galactomannan — The galactomannan (1 g) was hydrolyzed with 0 33N sulphuric acid (75 ml) for 3 h on a boiling water-bath The hydrolyzate was neutralized (barium hydroxide and barium carbonate) to pH 5, filtered, and deionized with Amberlite IR-45 (OH^-) and Amberlite IR-120 (H^+) resins The eluate containing the neutral sugars was then adsorbed on a column of silica gel–charcoal (4 1) and eluted with ethanol[30] Examination by paper chromatography (solvent *A*) revealed the presence of five oligosaccharides (R_F 0 47, 0 45, 0 34, 0 22, and 0 19), in addition to galactose and mannose Application of French and Wild's relationship[11] gave the results shown in Fig 1

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THE GLYCOSAMINOGLYCANS OF HUMAN TERM PLACENTA*

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ABSTRACT

The glycosaminoglycans of human term placenta have been extracted and analyzed Approximately one third of the total content may be extracted with saline, a 0 9% sodium chloride solution, and consists of hyaluronic acid, chondroitin 4-sulfate and chondroitin 6-sulfate (both at various levels of sulfation), and of a small proportion of dermatan sulfate and heparan sulfate. The residual glycosaminoglycans may be extracted after proteolytic digestion of the tissue, and consist mainly of heparan sulfate and dermatan sulfate, with a smaller proportion of chondroitin 4-sulfate It is postulated that the two hyaluronidase-resistant glycosaminoglycans may constitute a barrier to the passage of immunocompetent cells from the mother to the fetus

INTRODUCTION

In a recent article, Currie and Bagshawe[1] have suggested that the placenta may perform the function of a barrier which " prevents the host lymphocytes from making cell-to-cell contact with trophoblast" This unique property has been ascribed to the presence of sialomucins, capable of forming an electrochemical barrier to immunocompetent cells The presence of an amorphous, electron-dense material which could be stained with the periodic acid-Schiff reagent and with Hale colloidal iron has been described along the fetal-maternal interface[2]; more recently, the presence of glycosaminoglycans in human and mouse placenta and the variation of their content at various stages of pregnancy have been studied by Lovell, Tighe, and Curran[3], and by Svejcar[4].

This investigation describes the isolation and analytical data of various glycosaminoglycans present in human term-placenta.

EXPERIMENTAL

Extraction of glycosaminoglycans. — Four normal human placentas derived

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from full-term pregnancies were obtained from the Obstetric Service of St Joseph's Hospital, Houston, Texas The umbilical cords and the fetal membranes were removed, and excessive blood, blood clots, and serum were squeezed out The tissue (1 503 kg, wet weight) was homogenized with acetone (6 l) in a Waring Blendor and kept in acetone overnight at 4°. The insoluble material was collected by filtration, and air dried (251 5 g) After the visible vessel fragments had been removed by hand, the dry material was extracted once with a 0 9% sodium chloride solution (2 l) in a Waring Blendor The residue was collected by centrifugation (Sorvall RCB 2 centrifuge, GSA rotor, 15,000 r p m for 30 min, at 4°) and extracted repeatedly with a 0 9% sodium chloride solution using a Willems Polytron ultrasonic disintegrator, Model PT20, OD type, for 30 sec each time The extraction was discontinued when the 0 9% sodium chloride extracts became colorless The residue was collected by centrifugation, and the various extracts were combined and concentrated by lyophilization to a final volume of 2000 ml (placenta extract) The pH was adjusted to 8 50, pancreatin (1 g, Fisher, 5X) was added, and the extract was incubated for 48 h at 40°. The solution was boiled, then cooled, the pH was adjusted to 4 50, crude papain (0 5 g, activated with 50mM cysteine and 50mM ethylenediaminetetraacetic acid) was added, and incubation at 40° was resumed for 48 h The papain was then denaturated by heat, the pH of the digest was adjusted to 8 20, and the material was digested with Pronase (5 mg, Calbiochem, B grade) for 24 h at 37°

The residue from the sodium chloride extraction (placenta residue, 618 g, wet weight) was homogenized in a Waring Blendor with 50mM sodium hydroxide (pH 8 50, 2 l) and digested first with pancreatin, then with crude papain as described previously Upon completion of the digestion, the residual protein was precipitated by addition to the digest of trichloroacetic acid until a 6% concentration was obtained The dark precipitate was collected by centrifugation, washed with 6% trichloroacetic acid several times, and discarded The supernatant and washes were combined, neutralized with M sodium hydroxide, and dialyzed for several days at 4° The retentate was concentrated *in vacuo* and precipitated by addition of 95% ethanol containing 10% potassium acetate (3 vol) The precipitate was washed with 95% ethanol, absolute ethanol, and dried with ether It was dissolved in water, and the pH of the solution was brought to 8 20 The material was digested with Pronase (2 mg) for 2 days at 37°.

Both Pronase digests (placenta extract and placenta residue) were clarified separately by centrifugation at 8000 $\times g$ for 15 min at room temperature, and each supernatant was stirred for 1 h with Dowex-50 (10 g, X-2, 200–400 mesh, H^+) The resin was removed by filtration and washed repeatedly with water, which was added to the respective filtrates. These were neutralized, concentrated, and the glycosaminoglycans were precipitated by addition of 95% ethanol containing 10% potassium acetate (3 vol.). The precipitates were dissolved in water, and the residual salts were removed by ultrafiltration through a UM-1 Diaflo membrane (Amicon Corp., Lexington, Mass) After lyophilization of the retentates, 260 9 mg of material was obtained from the placenta extract and 438 6 mg from the placenta residue

Identification of glycosaminoglycans — The various analytical procedures used in this investigation have been described in detail in previous publications[5,6] Infrared spectra were recorded with a Beckman IR-9 spectrophotometer on material (10–20 μg) dried on an Irtran plate (Beckman Instruments, Inc). Chondroitin 4-sulfate was identified by absorption bands at 925 and 850 cm^{-1} and chondroitin 6-sulfate by bands at 775, 820, and 992 cm^{-1}

Aliquots (1 mg) of placenta extract and placenta residue were dissolved in distilled water (10 ml) and used for analyses (Tables I and II). A sample (200 mg) of each material was dissolved in a small volume of water, and the solution was applied to a column (21 × 2 cm) of Dowex-1 (X-2, 200–400 mesh, Cl^-) The columns were washed with distilled water (250 ml), and then eluted with a solution of sodium chloride (250 ml) of increasing molarity (0 2, 0 4, 0 6, 0 8, 1.0, 1 2, 1 4, 1 6, 1 8, 2 0, 3.0, and 4 0M, fractions I–XIII)[7] The hexuronic acid content of each eluate was measured with the borate–carbazole reaction[8] (Fig 1) Each eluate was desalted and concentrated by ultrafiltration through a UM-1 Diaflo membrane, and then lyophilized, and the residue weighed Aliquots of each dry eluate were used for analyses (Tables I and II)

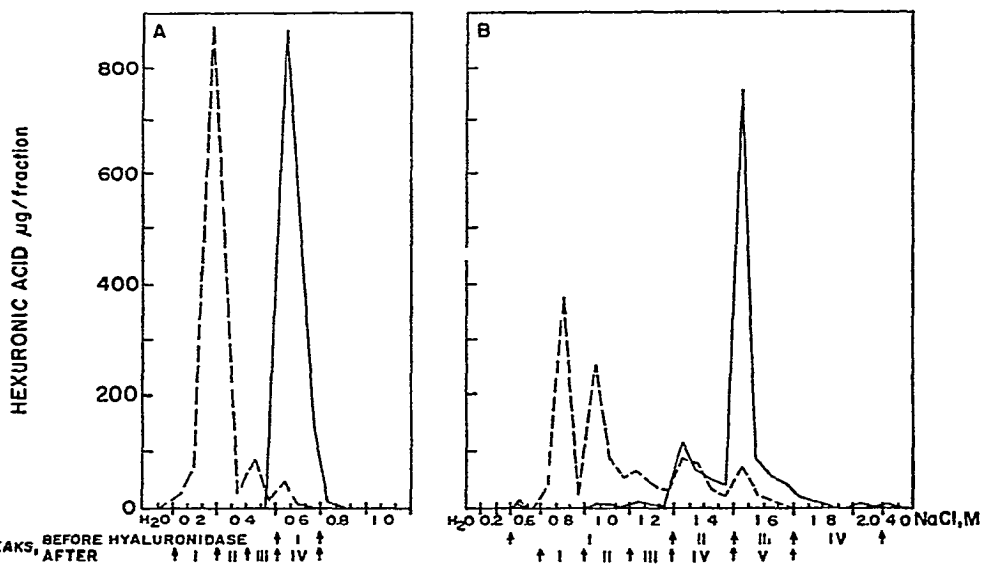


Fig 1 Fractionation on Dowex-1 (X-2) of fractions IV (A) and IX (B) of the placenta extract, before (———) and after (— — —) treatment with hyaluronidase The elution with a given concentration of sodium chloride was continued as long as hexuronic acid-containing material was eluted from the column Recoveries were quantitative (98–107%) The eluates were divided into arbitrary peaks as indicated.

The major fractions obtained from the placenta extract (0 6M and 1 6M sodium chloride) and from the placenta residue (0 8M and 1 6M sodium chloride) were investigated further. Approximately 4 mg of each fraction was dissolved in 0 1M acetate

buffer (4 ml, pH 4.9) which was 0 15M in sodium chloride Each solution was divided into two parts, and one of these was incubated with bovine, testicular hyaluronidase (1 mg, 300 USP U) for 48 h at 37°. Thereafter, digested and nondigested corresponding aliquots were each applied to a column (10 × 0.9 cm) of Dowex-1 (X-2, 200–400 mesh, Cl^-), and each column was eluted with a solution of sodium chloride of increasing concentration The recovery of hexuronic acid from the various columns was quantitative, and the various fractions obtained from each column were desalted by ultrafiltration, lyophilized, and the residue was analyzed The anionic oligosaccharides could be retained on UM-2 Diaflo membranes, provided that the salt concentration of the original solution was below 50mM

RESULTS

The yields of glycosaminoglycans obtained from human placenta with the technique described indicate that only a portion of them (placenta extract, less than one third of the total) may be extracted with a 0 9% sodium chloride solution prior to proteolytic digestion Chromatographic separation on Dowex-1 of the glycans extracted before and after proteolytic digestion (placenta extract and placenta residue) showed essentially the same pattern of elution It consists of two, not completely resolved, major peaks, one being eluted between 0.6 and 1 0M sodium chloride, and the second between 1 4 and 1 8M sodium chloride However, the placenta extract contained a higher proportion of glycosaminoglycans eluted at low sodium chloride concentrations and a lower proportion eluted at high sodium chloride concentrations

Tables I and II summarize the analyses of the glycosaminoglycans isolated from placenta extract and residue, before and after fractionation on Dowex-1 columns. Whereas the recovery of the two materials was quantitative when calculated in terms of the hexuronic acid content of the various sodium chloride eluates, it was only between 78 and 81% when calculated in terms of dry weight. This discrepancy may be explained by some loss of glycosaminoglycans through UM-1 Diaflo membranes at high salt concentrations[9]

From the analytical data it is evident that fractions I and II obtained from the placenta extract and the placenta residue contain small amounts of glycosaminoglycans and are contaminated with large amounts of material which have not been removed by the treatment with Dowex-50 It is possible that part of the amino sugars found in these fractions represent some of the glycoproteins known to be present in placenta[1,2]

Fraction III contains hexuronic acid and amino sugars in nearly equimolar amounts, both glucosamine and galactosamine, and small amounts of sulfate being present The fraction derived from the placenta residue seems to have more extraneous material than does the corresponding one from placenta extract

Fraction IV of the placenta extract represents the initial portion of the first elution peak. Upon rechromatography on Dowex-1, it was eluted by 0 6M sodium chloride as a single peak (Fig 1A) which contained glucosamine, galactosamine,

sulfate, and traces of 2-deoxy-2-sulfoamino- and 2-amino-2-deoxyhexose After incubation with hyaluronidase and chromatography on Dowex-1, the material was eluted at sodium chloride concentrations lower than 0 6M, only 3 5% of the original material still being eluted by 0 6M sodium chloride (Fig 1A) The results of analysis of the four arbitrarily divided peaks (Table III) indicate that the bulk of the depolymerized material (Peaks I and II) contained equimolar amounts of hexuronic acid and amino sugars, the latter including both glucosamine and galactosamine Since the i.r. spectra of these peaks show an absence of absorbance at 1230 cm^{-1} and a pattern similar to that obtained with hyaluronic acid preparations from bovine vitreous humor (Nutritional Biochemical Corp , Cleveland, Ohio, Worthington Biochemical Corp , Freehold, N J), it may be concluded that the material consists of hyaluronic acid and of nonsulfated or desulfated galactosamine-containing glycosaminoglycans

Fractions V and VI of the placenta extract complete the first elution peak. The analytical data (Table I) indicate the presence of glucosamine- and galactosamine-containing, scarcely sulfated glycosaminoglycans These may be identified, in part, as heparan sulfate, because of the presence of 2-deoxy-2-sulfoamino- and 2-amino-2-deoxyhexose and of the ratios of the colors obtained in the borate–carbazole reaction[8], and in part as chondroitin 4-sulfate, because of the infrared absorption at 925 and 850 cm^{-1}

Fraction VII placenta extract contains glucosamine and galactosamine The i r spectrum indicates the presence of both 4-sulfate and 6-sulfate esters The results of the colorimetric determinations are compatible with the presence of small amounts of heparan sulfate and large amounts of chondroitin 4-sulfate

Fractions VIII, IX, and X of the placenta extract represent the second elution-peak of the placenta extract Fully sulfated, galactosamine-containing glycosaminoglycans predominate, the i r spectra indicate the presence of 4-sulfate groups and, to a lesser extent, of 6-sulfate groups The ratios of the intensities of the colors obtained in the borate–carbazole and in the carbazole–naphthoresorcinol[10] reactions suggest a predominance of chondroitin 4-sulfate and a gradual increase of dermatan sulfate Repeated chromatography of fraction VIII on Dowex-1, before and after incubation with hyaluronidase (Fig 1 B), confirms this assumption After incubation with hyaluronidase, peaks I and II, which were eluted at sodium chloride concentrations lower than 1 6M (Table IV), produced colorimetric reactions suggesting the presence of chondroitin 4-sulfate and dermatan sulfate The colorimetric determination of peaks IV and V, however, suggested the preponderant presence of dermatan sulfate The different sensitivities of chondroitin 4-sulfate and dermatan sulfate to the action of testicular hyaluronidase[11] are in agreement with the observed different chromatographic behavior of their products of hydrolysis

Fraction IV of the placenta residue contains both glucosamine and galactosamine and traces of sulfate The presence of 2-deoxy-2-sulfoamino- and 2-amino-2-deoxyhexose indicates that part of the glucosamine is present as heparan sulfate

[*text continued p 546*]

TABLE I

FRACTIONATION ON DOWEX-1 OF PLACENTA EXTRACT

Fraction	*Eluent*	*Dry weight, mg*	*Hexuronic acid*[a] *(borate test)*	*Glucosamine*[a]	*Galactosamine*[a]	*Sulfur*[a]	*2-deoxy-2-sulfoaminohexose*[a]	*2-deoxy-2-aminohexose*[a]	*Ratio of absorbances*	
									Borate to carbazole test	*Carbazole to naphthoresorcinol test*
I	H_2O	1 2	1 15 (4 9)	0 60 (2 3)	0 40 (1 6)	negative				
II	0 2M NaCl	2.5	0 70 (11 7)	0 61 (9 4)	0 38 (6 0)	negative			2.50	3.60
III	0.4M NaCl	2 9	1 28 (22 5)	0 38 (6 7)	0 62 (10 0)	0 01 (0 03)			2 01	5.80
IV	0 6M NaCl	29 2	1 28 (41 7)	0 56 (16.9)	0 44 (13 1)	0 04 (0 24)	0 008 (0 24)	0 010 (0.29)	2.00	7 00
V	0.8M NaCl	28 1	1 32 (38 9)	0 67 (19.0)	0 33 (9 3)	0 28 (1 41)	0 104 (2 98)	0 033 (0 97)	1 91	7 93
VI	1 0M NaCl	13 4	1 25 (26 6)	0 66 (13 0)	0 34 (6 6)	0.58 (2 05)	0 302 (5.90)	0 038 (0 79)	1 93	9,39
VII	1 2M NaCl	9.7	1 05 (30 3)	0 26 (6 9)	0.74 (19 9)	0 78 (3 73)			1.72	9 23
VIII	1 4M NaCl	23 1	1 07 (26 3)	0 02 (0 6)	0 98 (22 3)	1 00 (4 12)			1 82	5 72
IX	1 6M NaCl	33 9	1 10 (32 2)	0 02 (0 6)	0 98 (26 3)	1 12 (5 40)			2 03	3.80
X	1 8M NaCl	7.8	1 06 (28 5)	0 03 (0.7)	0 97 (24.0)	1 22 (5.44)			2 42	1.71
XI	2.0M NaCl	5.6	0 97 (18 9)	0 02 (0.5)	0 98 (17 6)	1 05 (3 40)			2 47	1 19
XII	3.0M NaCl	5.3	0 98 (23 7)	0 04 (1.0)	0 96 (21 4)	1 04 (4 16)			2.51	1 08
XIII	4 0M NaCl	1 1	1 24 (4 5)	0 16 (0 5)	0 84 (2.8)	2 12 (1 30)			2 40	1 15
Placenta extract before fractionation			1 13 (31.9)	0 34 (8.8)	0 66 (17 1)	0.69			1.99	4.00

[a]Analytical results are expressed as ratios of atom or residue to hexosamine residue, taken as 1 00 Figures in parentheses are per cent values.

TABLE II

FRACTIONATION ON DOWEX-1 OF PLACENTA RESIDUE

Fraction	*Eluent*	*Dry weight, mg*	*Hexuronic acid*[a] *(borate test)*	*Glucosamine*[a]	*Galactosamine*[a]	*Sulfur*[a]	*2-deoxy-2-sulfoaminohexose*[a]	*2-deoxy-2-aminohexose*[a]	*Ratio of absorbances*	
									Borate to carbazole test	*Carbazole to naphthoresorcinol test*
I	H_2O	3 2	1 00 (5 1)	0 65 (3.0)	0 35 (1.7)	negative			2.60	6 40
II	0.2M NaCl	5 8	0 97 (11 8)	0 63 (7 1)	0 36 (4 3)	negative			2 07	4.60
III	0 4M NaCl	6.9	1 20 (12 7)	0 50 (4 9)	0 50 (4 8)	0 17 (0 28)			2.48	3 47
IV	0 6M NaCl	10 7	1.27 (23 4)	0 48 (8 1)	0 52 (8.8)	0 24 (0 73)	0 048 (0 82)	0 021 (0 35)	1 99	7 40
V	0 8M NaCl	20 7	1 53 (32 7)	0 87 (17 1)	0 13 (2.6)	0 49 (1 73)	0.210 (4 16)	0 048 (0 96)	1 69	6 50
VI	1 0M NaCl	11 0	1 45 (35.1)	0 54 (12.0)	0 46 (10 2)	0 51 (2 02)	0 223 (4 98)	0 044 (1 00)	1.81	6 60
VII	1 2M NaCl	10 0	1 39 (29 6)	0 27 (5 4)	0 73 (14 1)	0 69 (2.41)			2 02	5 06
VIII	1.4M NaCl	15 2	1.18 (30 3)	0 06 (1.5)	0 94 (22 1)	0.89 (3 76)			2.17	3 66
IX	1 6M NaCl	56.3	0 99 (22 8)	0 02 (0 4)	0 98 (21 6)	1.05 (4 11)			3.07	0 71
X	1 8M NaCl	5 5	0.91 (9 8)	0 09 (0.9)	0 91 (9.1)	1 40 (2 51)			2 69	0 62
XI	2 0M NaCl	1 7	0 87 (12 8)	0 09 (1 4)	0 91 (12 4)	1 41 (3 43)			2 71	0 62
XII	3 0M NaCl	2 5	0 86 (11.5)	0 11 (1 3)	0 89 (11 0)	3 13 (6 83)			2.78	0 65
XIII	4 0M NaCl	0 7	1 07 (3 2)	0 27 (0 8)	0 73 (2.1)	2 80 (1 44)			2 77	1 10
Placenta residue before fractionation			1 22 (24 8)	0 25 (4.7)	0 75 (18 7)	0 89			2 32	2 18

[a]See Table I.

TABLE III

FRACTIONATION ON DOWEX-1 OF FRACTION IV OF THE PLACENTA EXTRACT, BEFORE AND AFTER TREATMENT WITH HYALURONIDASE

Fraction	*Eluent*	*Hexuronic acid*		*Glucosamine*[b]	*Galactosamine*[b]	*Ratio of absorbances*	
		%[a]	*Borate test*[b]			*Borate to carbazole test*	*Carbazole to naphthoresorcinol test*
Before hyaluronidase treatment							
Peak I	0.6M NaCl	97 1	1 23	0 52	0 48	2 00	9 7
After hyaluronidase treatment							
Peak I	0 2M NaCl	62 9	1 02	0 56	0,44	2 17	8 18
Peak II	0.4M NaCl (first half)	31 5	1 06	0 47	0 53	2 00	5 38
Peak III	0 4M NaCl (second half)	6 6	1 08	0 30	0 70		
Peak IV	0.6M NaCl	3 5	1 21	0 85	0 14		

[a]Per cent recovery based on total hexuronic acid applied to the column [b]Analytical results expressed as ratios of atom or residue to hexosamine residue, taken as 1 00

TABLE IV

FRACTIONATION ON DOWEX-1 OF FRACTION IV OF THE PLACENTA EXTRACT, BEFORE AND AFTER TREATMENT WITH HYALURONIDASE[a]

Fraction	*Eluent*	*Hexuronic acid*		*Glucosamine*	*Galactosamine*	*Ratio of absorbances*	
		%[a]	*Borate test*			*Borate to carbazole test*	*Carbazole to naphthoresorcinol test*
Before hyaluronidase treatment							
Peak I	0 6–1 2M NaCl	2.7	1 09	0 37	0 63	1 65	4 48
Peak II	1.4M NaCl	20 9	1.02	0 02	0 98	1 92	2 82
Peak III	1.6M NaCl	73.2	1.16	0.01	0 99	2.31	1.49
Peak IV	1 8–2.0M NaCl	2.8	1.13	0 05	0 95	2 30	0 96
After hyaluronidase treatment							
Peak I	0 8M NaCl	35 7	0.96	0.03	0 97	1 81	5.9
Peak II	1 0M NaCl	32 3	1.11	0 02	0 98	1 86	3.26
Peak III	1 2M NaCl	10.4	1.08	0 02	0.98	2 47	0.92
Peak IV	1 4M NaCl	18 3	0 96	0.03	0 97	3 32	0 42
Peak V	1.6M NaCl	9 3	1 03	0 03	0.97	3.31	0.42

[a]See Table III

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TABLE V

FRACTIONATION ON DOWEX-1 OF FRACTION V OF THE PLACENTA RESIDUE, BEFORE AND AFTER TREATMENT WITH HYALURONIDASE[a]

Fraction	*Eluent*	*Hexuronic acid*		*Glucosamine*	*Galactosamine*	*Ratio of absorbances*	
		%[a]	*Borate test*			*Borate to carbazole test*	*Carbazole to naphthoresorcinol test*
Before hyaluronidase treatment							
Peak I	0.6M NaCl	13.8	1.26	0.72	0.28	1.65	6.1
Peak II	0.8M NaCl	79.5	1.52	0.88	0.12	1.67	6.5
Peak III	1.0M NaCl	4.7	1.50	0.79	0.21	1.71	
After hyaluronidase treatment							
Peak I	0.2–0.4M NaCl	6.6	1.05	0.11	0.89	1.84	8.0
Peak II	0.6M NaCl	21.2	1.25	0.92	0.08	1.69	9.0
Peak III	0.8M NaCl	68.3	1.64	0.96	0.04	1.66	7.0
Peak IV	1.0M NaCl	3.0	1.37	0.89	0.10	1.71	

[a]See Table III

TABLE VI

FRACTIONATION ON DOWEX-1 OF FRACTION IX OF THE PLACENTA RESIDUE, BEFORE AND AFTER TREATMENT WITH HYALURONIDASE[a]

Fraction	*Eluent*	*Hexuronic acid*		*Glucosamine*	*Galactosamine*	*Ratio of absorbances*	
		%[a]	*Borate test*			*Borate to carbazole test*	*Carbazole to naphthoresorcinol test*
Before hyaluronidase treatment							
Peak I	1.4M NaCl	14.7	0.92	0.04	0.96	2.77	
Peak II	1.6M NaCl	80.8	0.92	0.00	1.00	3.36	0.62
After hyaluronidase treatment							
Peak I	0.6–0.8M NaCl	19.7	1.22	0.03	0.97	1.87	5.21
Peak II	1.0M NaCl	17.7	1.19	0.00	1.00	2.00	3.73
Peak III	1.2M NaCl	8.3	1.04	0.02	0.98	2.12	1.11
Peak IV	1.4M NaCl	20.1	0.97	0.01	0.99	3.16	0.51
Peak V	1.6M NaCl	46.1	1.00	0.02	0.98	3.88	0.38

[a]See Table III.

Fraction V of the placenta residue consists mainly of heparan sulfate, as suggested (Table II) by the abundance of glucosamine and by the ratio hexuronic acid to hexosamine[12]. This is confirmed by the presence of 2-deoxy-2-sulfoamino- and 2-amino-2-deoxyhexose, by the ratio of the intensity of the color obtained in the borate–carbazole reactions and by the chromatographic behavior on Dowex-1, before and after incubation with hyaluronidase As demonstrated in Fig 2A and in Table V, the latter treatment does not change the elution pattern of the material, except for the elution at very low salt concentration (0 2M sodium chloride) of a small amount of material containing preponderantly galactosamine

Fraction VI of the placenta residue is a mixture of heparan sulfate and galactosamine-containing glycosaminoglycans, probably chondroitin 4-sulfate, on the basis of the I R spectrum.

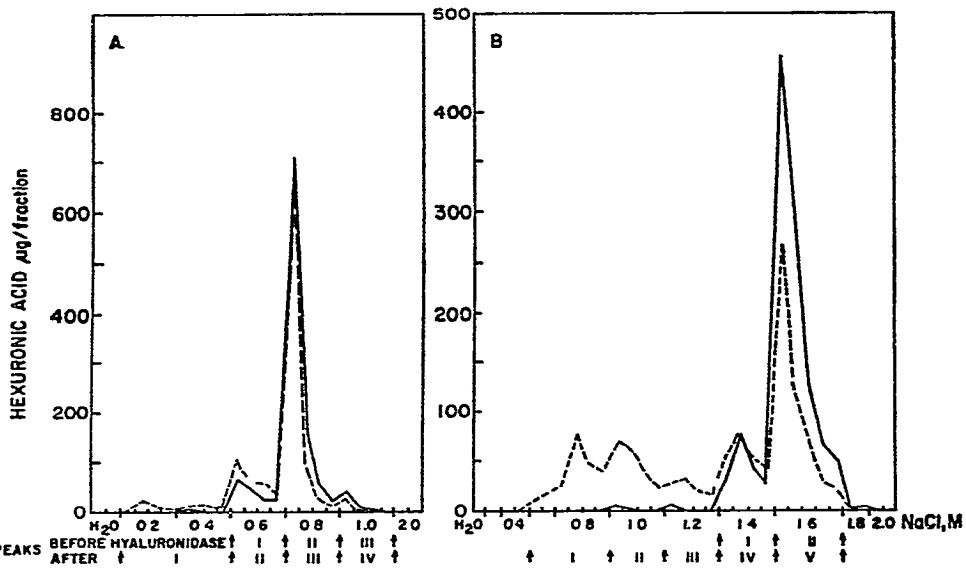


Fig 2 Fractionation on Dowex-1 (X-2) of fractions V (A) and IX (B) of the placenta residue, before (———) and after (— — —) treatment with hyaluronidase The elution with a given concentration of sodium chloride was continued as long as hexuronic acid-containing material was eluted from the column Recoveries were quantitative (98–112%) The eluates were divided into arbitrary peaks as indicated

Fractions VII and VIII of the placenta residue show an increasing proportion of galactosamine and sulfate The ratios of the intensities of the colors obtained in the borate–carbazole and carbazole–naphthoresorcinol tests suggest the presence of chondroitin 4-sulfate; this is confirmed by the I R spectra which also indicate the presence of a small proportion of 6-sulfate

Fractions IX and X of the sodium chloride placenta residue contain increasing amounts of dermatan sulfate, as indicated by the colorimetric determinations The chromatographic behavior on Dowex-1 of the 1 6M sodium chloride eluate, before

and after incubation with hyaluronidase, demonstrates the presence of considerable proportions of dermatan sulfate (Fig 2 B, Table VI), since only 40% of the original material is depolymerized by the enzyme, and eluted at sodium chloride concentrations lower than those required for the elution of the nondigested material

Fractions XI, XII, and XIII of the placenta extract and placenta residue represent mainly galactosamine-containing glycosaminoglycans, having a sulfate to hexosamine ratio higher than 1 The colorimetric determinations of fractions XI and XII of the placenta residue indicate the presence of dermatan sulfate, and the i r spectra are typical for 4-sulfate groups Unfortunately, the small amounts of material available did not allow further purification and enzymic study of these fractions It is worthy of mention that analyses of amino sugars performed with the amino acid analyzer revealed the presence of small amounts of hydroxylysine in the hydrolyzates of the various fractions of the placenta residue

DISCUSSION

Whereas the data obtained by Lovell, Tighe, and Curran[3] demonstrated the presence of hyaluronic acid, chondroitin 6-sulfate, and dermatan sulfate in a portion of placenta containing the terminal chorionic villi, our data indicate that chondroitin 4-sulfate and heparan sulfate also are present in the whole human placenta Thus, our results are in agreement with Svejcar's data[4], obtained from the study of mouse placenta

The extraction method used in our investigation demonstrates that only a portion of the glycosaminoglycans of the placenta may be extracted by homogenization in 0 9% sodium chloride solution This fraction (placenta extract) consists mainly of nonsulfated, glucosamine- and galactosamine-containing glycosaminoglycans, of chondroitin sulfate, and of small proportions of heparan sulfate and dermatan sulfate A larger fraction of the glycosaminoglycans of placenta may be extracted only after exhaustive proteolytic digestion (placenta residue), and it consists mainly of heparan sulfate and dermatan sulfate, with a minor proportion of chondroitin sulfate The presence of hydroxylysine in these fractions suggests the possibility that the latter glycosaminoglycans may be associated with collagen or basement membranes[13,14] Although it is known that dermatan sulfate is preferentially associated with coarse collagen fibers[15], it is likely that the large amounts of heparan sulfate found results from the abundance of vessels present in the organ

If this assumption is correct, then two negatively charged, electrochemical barriers would exist in the placenta one represented by large amounts of heparan sulfate and located at the level of the maternal sinuses and fetal, capillaries and the other, consisting mainly of dermatan sulfate, located in the mesenchymal tissue interposed between the maternal and fetal, vascular structures One would like to speculate that the presence of a double, hyaluronidase-resistant structure at the level of the maternal-fetal interface may assure a long-lasting and effective barrier to the migration of immunocompetent cells from mother to fetus

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SYNTHESIS OF THE TWO D-2-PENTULOSES NEW DERIVATIVES OF D-*erythro*-PENTULOSE*

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(Received February 10th, 1969)

ABSTRACT

The syrupy mixture of eight pentoses obtained by boiling a solution of either D-xylose (**1**) or D-arabinose (**2**) in pyridine for 4 5 h was freed of the four D-aldopentoses The resulting mixture of four D-pentuloses was resolved by condensation with acetone, followed by partition between solvents or by distillation, in this way, crystalline 2,3-*O*-isopropylidene-*β*-D-*threo*-pentulofuranose was readily prepared from **1**, and crystalline 1,2·3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose (**14**) from **2** Each of the isomerizations yielded a small proportion of the epimeric D-2-pentulose, but the proportions of the D-3-pentuloses were much lower The diacetal **14** was partially hydrolyzed, with dilute acid, to 3,4-*O*-isopropylidene-D-*erythro*-pentulofuranose (**17**), the α-D anomer of which was obtained in crystalline form The structure of **17** was proved by oxidation with periodate, to give the known, crystalline 2,3-*O*-isopropylidene-D-erythrono-1,4-lactone The anomers of the 1,3,4-triacetate and 1,3,4-tribenzoate of methyl D-*erythro*-pentulofuranoside were prepared, and characterized by specific optical rotation and n m r spectroscopic evidence

GENERAL INTRODUCTION

Crystalline derivatives of D-*threo*-pentulose ("D-xylulose") and D-*erythro*-pentulose ("D-ribulose") were desired for issuance by the National Bureau of Standards as Standard Reference Materials We have therefore had occasion to synthesize fairly large quantities of these sugars, this entailed repetition of some of the earlier work[1], and we have now made certain modifications that simplify and improve the preparation and isolation of the two D-2-pentuloses

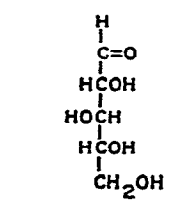
Part I *Improved preparation of 2,3-*O*-isopropylidene-β-*D-threo*-pentulofuranose* (13)

INTRODUCTION

D-*threo*-Pentulose (**5**) is of considerable interest to biochemists, as it is probably

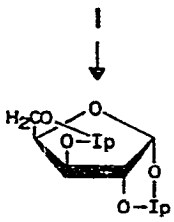
*Presented, in part, at the Fourth Middle Atlantic Regional Meeting of the American Chemical Society, Washington, D C, February 14, 1969

an intermediate in the photosynthetic cycle[2] It was synthesized[3] in 1933 by the pyridine-catalyzed isomerization of D-xylose (**1**) Other nonenzymic methods for its preparation include the action of bromine on 1-deoxy-1-*p*-toluidino-D-lyxitol[4], a 5-step synthesis from D-arabinitol[5], and a 5-step synthesis from 2,3,5-tri-*O*-benzyl-D-arabinose[6]. [Nonenzymic methods used for preparation of the enantiomorph (**5-e**), the pentulose involved in human pentosuria[7], are the pyridine-catalyzed isomerization of L-xylose[8] and an 8-step synthesis from L-ascorbic acid[9]]

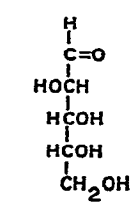


D-Xylose

1

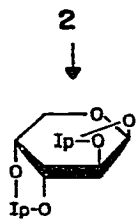


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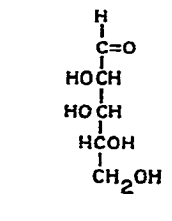


D-Arabinose

2

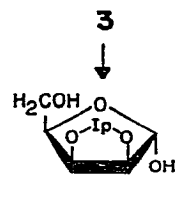


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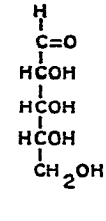


D-Lyxose

3

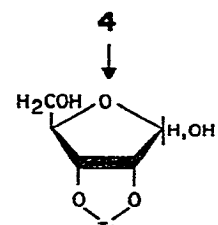


11

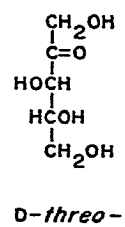


D-Ribose

4

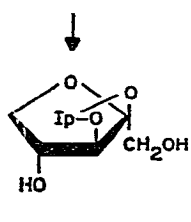


12



D-*threo*-Pentulose

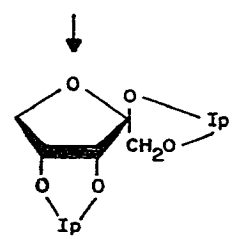
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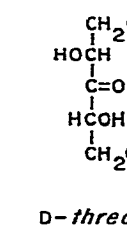
13

D-*erythro*-Pentulose

6

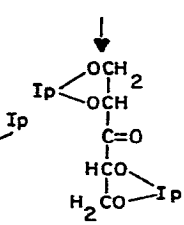


14

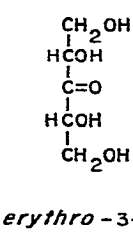


D-*threo*-3-Pentulose

7

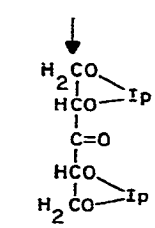


15



erythro-3-Pentulose

8



16

The preparative value of the pyridine-catalyzed reaction was improved by Levene and Tipson[1], who acetonated the mixture of pentoses that is formed, and separated the resulting mixture of mono- and di-isopropylidene acetals by fractional distillation followed by crystallization They thus isolated four acetal fractions, namely, crystalline 1,2 3,5-di-*O*-isopropylidene-α-D-xylofuranose (**9**) derived from unchanged **1**, syrupy 2,3-*O*-isopropylidene-α-D-lyxofuranose* (**11**), crystalline 2,3-*O*-isopropylidene-β-D-*threo*-pentulofuranose** (**13**), and a syrupy mixture (*A*) of unidentified di-*O*-isopropylidenepentoses (free from **9**) having n_D^{25} 1 4414 and $[\alpha]_D^{25}$ −47.8° (acetone)

On complete hydrolysis with 100 mM oxalic acid, mixture *A* gave a mixture of pentoses having $[\alpha]_D^{25}$ −5.7° (calculated as pentose; in water) These properties suggested that mixture *A* consisted mainly of 1,2·3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose (**14**), the enantiomorph (**14-e**) of which had been shown by Levene and Tipson[1] to have n_D^{25} 1 4422 and $[\alpha]_D^{27}$ +105 5° (acetone), and to give, on hydrolysis, L-*erythro*-pentulose (**6-e**) having $[\alpha]_D^{27}$ +16 6° (water).

RESULTS AND DISCUSSION

For the isomerization, the concentration of the solution of D-xylose in pyridine has been doubled, affording a higher yield of the products and permitting the isomerization of a larger amount of D-xylose per preparation Use of absolute ethyl alcohol, instead of 95% ethyl alcohol, for removal of unchanged D-xylose enhanced the proportion of the latter that could be removed from the reaction mixture prior to acetonation.

As before[1], the monoacetals were separated from the diacetals by fractional distillation After crystallization and removal of 2,3-*O*-isopropylidene-β-D-*threo*-pentulose (**13**) from the monoacetal fraction, careful refractionation of the liquid remaining permitted the isolation of crystalline 2,3-*O*-isopropylidene-α-D-lyxofuranose (**11**), whereas in the earlier work[1], this fraction had not been obtained crystalline, even on nucleation with authentic **11**

Similarly, as much of **9** as possible was removed from the diacetal fraction by crystallization of **9** and filtration. The filtrate was fractionally redistilled, and sufficient of the unknown di-*O*-isopropylidenepentose fraction *A*, previously encountered[1], was accumulated to permit its further study, it was treated with 100 mM oxalic acid for 40 min at 25°, and the unhydrolyzed acetal was isolated, and identified as 1,2 3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose, the β-D anomer (**14**) of which is depicted by analogy with the structure of 1,2 4,5-di-*O*-isopropylidene-β-D-fructopyranose†, which likewise has a spiran structure at C-2.

*The precise structure of compound **11**, first crystallized and reported by Levene and Tipson[1], has since been proved[10]

**This structure was proved by Levene and Tipson[11]

†This anomeric configuration has been proved by X-ray crystallographic study (R D Rosenstein and S Takagi, personal communication, February 5th, 1969)

After completion of this work*, Fedoroňko and Linek[13] re-examined the mixture of pentoses obtained by the action (8 h) of boiling pyridine on D-xylose By use of paper and column chromatography, they found that, in addition to the pentoses already mentioned (**1**, **3**, **5**, and **6**), it contains D-arabinose (**2**), D-ribose (**4**), D-*threo*-3-pentulose (**7**), and *erythro*-3-pentulose (**8**), all in unstated proportions

Consequently, in restudying the reaction with a view to increasing the yield of acetal **13**, it was decided to remove the aldopentoses (**1**, **2**, **3**, and **4**) from the mixture of isomers (after crystallization and filtration of unchanged **1**) Such removal of an aldose from a solution of an aldose and a ketose was achieved in 1950 by Gabrielson and Samuelson[14] by use of Amberlite IRA-400 (HSO_3^-) ion-exchange resin, and the method has since been improved[15]. Dowex-1 (HSO_3^-) ion-exchange resin was used by Lindberg and Slessor[16] for similar separation of the aldopentoses from the pentuloses obtained from D-xylose, they found that the pentulose mixture consisted of D-*threo*-pentulose and (for reaction times >8 h) "unidentified compounds (other ketoses?)".

D-Xylose (**1**) was therefore isomerized as already described, and, after removal of much **1** by crystallization, the mixture of pentoses was passed through a column of Amberlite IRA-400 (HSO_3^-) which had been prepared as described by Adachi and Sugawara[17] (see also, ref. 18), and the mixture of pentuloses was eluted and treated as described by Zinner and Rehpenning[18] The resulting mixture of compounds **5**, **6**, **7**, and **8** was acetonated, giving a mixture of **13**, **14**, **15**, and **16** as a syrup that contained a preponderance of **13**, because, without distillation of the syrup, the monoacetal **13** crystallized from it, affording an almost solid mass of crystals The monoacetal **13** was readily separated from the diacetals (**14**, **15**, and **16**) by distillation, or by dissolving the mixture of acetals in water and extracting with ether, in the latter separation, **13** remains in the aqueous layer, and the mixture of the three diacetals is obtained in the ether layer If the isomerization with pyridine is conducted for only 4 5 h, these diacetals constitute a minor by-product

Part II *Improved preparation of 1,2 3,4-di*-O-*isopropylidene*-D-erythro-*pentulofuranose* (**14**)

INTRODUCTION

D-*erythro*-Pentulose (**6**) is a hitherto rare sugar that is an intermediate in the metabolism of D-glucose in animals[19], and it is an early product of photosynthesis in plants[20] It was synthesized[21] in 1934 by the pyridine-catalyzed isomerization of D-arabinose (**2**), and isolated as the crystalline (*o*-nitrophenyl)hydrazone[22] in 1935 Nonenzymic methods for its preparation include an 8-step synthesis[23] (of its 1,3,4,5-tetrabenzoate) from D-glucose (compare ref 24), a 4-step synthesis of the 3,4-*O*-isopropylidene-1,5-di-*O*-trityl derivative of **6** from benzyl 3,4-*O*-isopropylidene-*β*-D-

*A brief account was given in ref 12

arabinoside[25], formation (in a yield of 1 5%) by treatment of methyl *β*-D-*ribo*-hexopyranosid-3-ulose with dilute sulfuric acid[26], and decarboxylation of D-*arabino*-hexulosonic acid with nickel acetate in pyridine[27]

The enantiomorph (**6-e**) was prepared[21] in 1934 by the pyridine-catalyzed isomerization of L-arabinose (**2-e**), and, two years later, Levene and Tipson[1] improved the preparative value of the reaction by acetonating the mixture of pentoses that is formed, and separating the resulting mixture of acetals by fractional distillation In this way, they isolated crystalline 1,2 3,4-di-*O*-isopropylidene-*β*-L-arabinose (**10-e**) (derived from unchanged **2-e**), crystalline 1,2 3,4-di-*O*-isopropylidene-L-*erythro*-pentulofuranose (**14-e**), and syrupy 2,3-*O*-isopropylidene-L-ribofuranose (**12-e**), and obtained indications of the presence of other pentose mono- and di-acetals that were not identified

Over 30 years later, Fedoroňko and Linek[13] re-examined the mixture of pentoses obtained on treatment (4 h) of D-arabinose (**2**) By use of paper and column chromatography, they found that **2** gives a mixture of the same pentoses as they obtained[13] from D-xylose (**1**) (Obviously, however, the proportions of the pentoses in the mixture will be different, starting with **1** or **2**, unless the reaction is permitted to proceed to equilibrium in both instances) D-Arabinose (**2**) gave a mixture in the following proportions (by weight) D-xylose (?), D-arabinose (?), D-lyxose (1 0), D-ribose (16 7), D-*threo*-pentulose (5 2), D-*erythro*-pentulose (14 1), D-*threo*-3-pentulose (2 3), and *erythro*-3-pentulose (1 4) Later, Zinner and Rehpenning[18] studied the mixture of pentoses obtained from **2**, they separated the aldopentoses from the pentuloses by passing a solution of the mixture through a column of Wofatit SBW (HSO_3^-) ion-exchange resin, which retained the aldoses They stated that the effluent contained mainly D-*erythro*-pentulose (**6**), together with traces of *two* other pentuloses, but they used this mixture for the direct preparation of materials alleged to be derivatives of **6** [Starting with the enantiomorph (**2-e**), the resulting crude **6-e** was also found to contain other pentuloses[16]]

RESULTS AND DISCUSSION

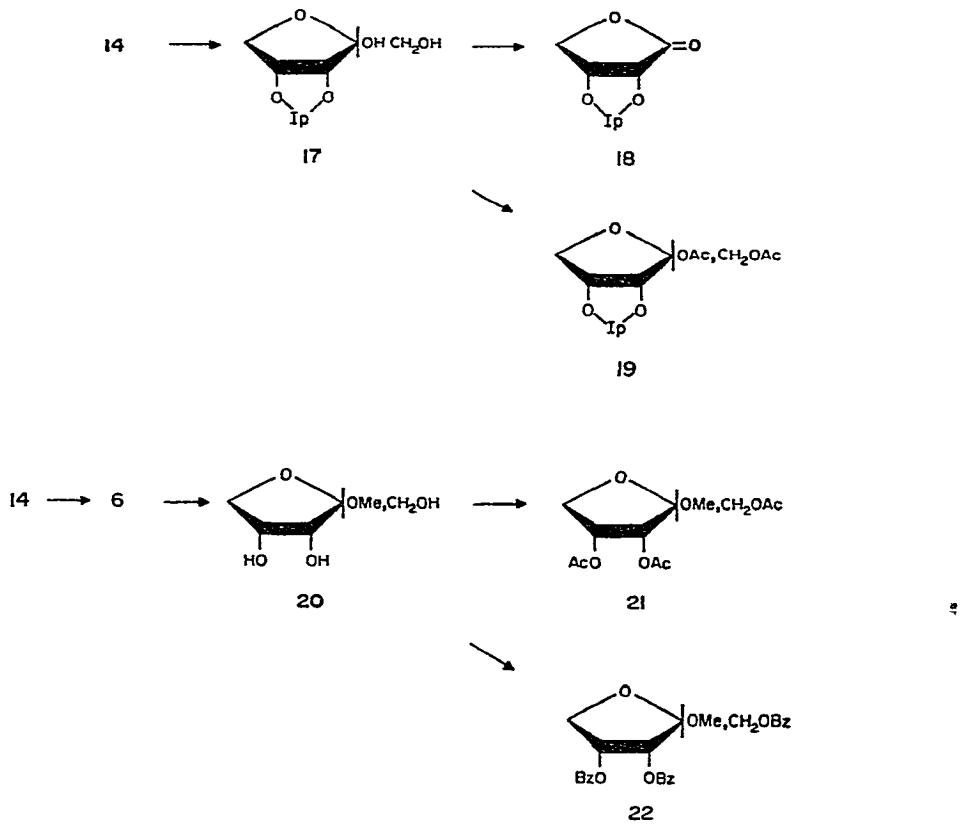
In order to prepare relatively large quantities of D-*erythro*-pentulose (**6**), we decided not to employ the inexpensive D-xylose as the starting material, because of the relatively low yield of **6** obtained therefrom. Instead, we chose to (*a*) apply Levene and Tipson's procedure[1] to D-arabinose (**2**), (*b*) remove the aldopentoses (**1**, **2**, **3**, and **4**) from the initial reaction mixture (after removal of as much as possible of **2** by crystallization), and then (*c*) acetonate the mixture of pentuloses (**5**, **6**, **7**, and **8**), and separate the mixture of one monoacetal and three diacetals as already described for the mixture from D-xylose It was found that, whereas analytically pure 2,3-*O*-isopropylidene-*β*-D-*threo*-pentulofuranose (**13**) could be isolated either by solvent partition or by fractional distillation followed by solvent partition, pure 1,2 3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose (**14**) is obtainable only by distillation, because the small content of diacetals **15** and **16** in the diacetal fraction is not removable by the solvent partition described

As Zinner and Rehpenning[18] had described 13 compounds prepared from crude D-*erythro*-pentulose (that must also have contained the pentuloses **5**, **7**, and **8**), we have prepared a sample of pure D-*erythro*-pentulose (**6**) by complete hydrolysis of distilled, crystalline diacetal **14**, and have condensed the free sugar with (*p*-tolylsulfonyl)hydrazine. The properties of the resulting (*p*-tolylsulfonyl)hydrazone differed from those reported by Zinner and Rehpenning[18] for the compound

The diacetal **14** is completely hydrolyzed to D-*erythro*-pentulose (**6**) by 100 mM oxalic acid in 56 h at 25° and in 72 min at 65°; in contrast, with 4 1 (v/v) acetic acid–water as the hydrolyst, treatment for 8 h at 65° is required. In a polarimetric study of the rates of these hydrolyses, the optical rotation decreased steadily from the high value for **14** to the low (equilibrium) value for **6**, with no intermediate maximum or minimum; thus, polarimetric observation affords no evidence as to the intermediate formation or nonformation of a monoisopropylidene acetal of D-*erythro*-pentulose Consequently, the hydrolysis was followed by t l c , and it was found that a monoacetal is, indeed, formed The concentration of **14** decreases steadily, while that of **6** increases steadily, and the concentration of the monoacetal rapidly rises to a moderate value that is maintained until, near the end of the hydrolysis, the compound disappears With this information, it then became possible to arrest the hydrolysis after the elapse of sufficient time to afford the optimal yield of monoacetal with minimal hydrolysis of the diacetal The acetals were then separated from free **6** by extraction with ether, and the monoacetal was separated from the diacetal by extraction of the ether solution with water

Purification of the monoacetal by distillation gave a syrup, $[\alpha]_D^{25}$ −63 0° (acetone), that was homogeneous by t l c. On slow crystallization from ether, one anomer, having $[\alpha]_D^{25}$ −5.4° (acetone), was isolated. Both the crystalline material and the syrup reduced Benedict solution rapidly, and both showed mutarotation in water, to give the same equilibrium rotation; consequently, one of the two free hydroxyl groups in the monoacetal is attached to C-2, the anomeric carbon atom To determine the position of attachment of the other hydroxyl group, the monoacetal was oxidized with sodium metaperiodate, the crystalline product was identified as the known[10] 2,3-*O*-isopropylidene-D-erythrono-1,4-lactone (**18**), showing that the free hydroxyl groups in the monoacetal are attached to C-1 and C-2 Hence, the monoacetal is 3,4-*O*-isopropylidene-D-*erythro*-pentulofuranose (**17**), and the diacetal from which it was prepared is, as would be expected, 1,2 3,4-di-*O*-isopropylidene-D-*erythro*-pentulofuranose (**14**). Moreover, the properties of **18** were identical with those of authentic **18**, showing that our sample of **14** was not contaminated by the corresponding diacetal of the enantiomorph of **6** (which might theoretically be formed in the isomerization with pyridine).

Compound **14** has two *spiro*-fused, 5 membered rings, namely, (*a*) the 1,3-dioxolane ring involving O-1, C-1, C-2, and O-2, and (*b*) the furanoid ring, it also has two *cis*-fused, 5-membered rings, namely, (*a*) the 1,3-dioxolane ring involving O-3, C-3, C-4, and O-4, and (*b*) the furanoid ring Evidently, the *spiro*-fused 1,3-dioxolane ring at the anomeric center is *more* susceptible to hydrolysis by acid than the 1,3-di-



oxolane ring *cis*-fused to the furanoid ring [This behavior resembles that of 1,2 5,6-di-*O*-isopropylidene-α-D-glucofuranose which, on hydrolysis* with 100 mM oxalic acid, loses the nonfused, 5,6-isopropylidene acetal group in 20 h at 20°, but retains the 1,3-dioxolane ring *cis*-fused (at C-1,C-2) to the furanoid ring] The hydrolysis of **14** contrasts[28] with that of 1,2 4,5-di-*O*-isopropylidene-D-fructopyranose, in which the *spiro*-fused 1,3-dioxolane ring is *less* susceptible to hydrolysis than the 1,3-dioxolane ring *cis*-fused at C-4,C-5 to a *pyranoid* ring

The optical rotation (in water) of the crystalline form of monoacetal **17** was more positive than that of the syrupy mixture of anomers, suggesting that the crystalline material is the α-D anomer This assignment is supported by the results of n m r. spectroscopy; the low value for the chemical shift of the C-1 protons (see Table I) in the spectrum of a solution of the crystals suggests that C-1 may be *trans* to the 1,3-dioxolane ring fused at C-3,C-4 In addition, the appearance of the signal of the C-1 protons as a singlet fits the pattern shown by the anomeric triesters **21** and **22** (see later)

On treatment with acetic anhydride–pyridine, both the crystalline and the

*Determination made by Barbara F. West

syrupy **17** were converted into a syrupy mixture, not yet crystallized, of two diacetates (**19**) that, by t l c, was found to contain the same two compounds, although in different proportions

Both the crystalline and the syrupy **17** were completely hydrolyzed to D-*erythro*-pentulose (**6**) by 4 1 (v/v) acetic acid–water in 1 h at 65° Acetylation or benzoylation of syrupy D-*erythro*-pentulose gave a syrupy mixture of esters, shown by t l c. to consist of three compounds, presumably the tetraesters of the acyclic form and of the two anomers of the furanose Therefore, in order to obtain a less complex mixture of esters that might crystallize, a mixture of the anomers of methyl D-*erythro*-pentulofuranoside (**20**) was prepared by condensation of **6** with methanol containing 1% of hydrogen chloride, the resulting mixture was acetylated or benzoylated, yielding, in each instance, a mixture of two compounds (as shown by t l c) Preparative t l c. of each mixture effected the isolation of each anomer, however, of the four compounds (**21**, α and β, **22**, α and β), only a methyl 1,3,4-tri-*O*-benzoyl-D-*erythro*-pentulofuranoside (probably the α-D anomer of **22**) has thus far been obtained in crystalline form

N m r data for the D-*erythro*-pentulose derivatives prepared are given in Table I. It is interesting that, for the esters **21** and **22**, the multiplicity of the signal due to the two protons on C-1 is quite definitely dependent on the configuration at the anomeric center (C-2) For the compounds presumed to be the α-D anomers, the protons on C-1 give rise to a singlet, whereas, for the compounds presumed to be the β-D anomers, the H-1 signals appear as two 1-proton doublets It seems reasonable to suppose that this multiplicity is a consequence of the restricted rotation of the methylene group (C-1) when it is *cis* to the 3-acyloxy group. These assignments are supported by the positions of the signals due to the anomeric methoxyl groups of the respective, acylated methyl glycosides, the signal appears at lower field for the presumed α-D anomers, in which the methoxyl group would be *cis* to the 3-acyloxy group These tentative assignments agree, in each instance, with the relative, specific optical rotation of the two anomers, and would therefore appear to permit unequivocal assignment of anomeric configuration to the anomers of compounds **21** and **22**

EXPERIMENTAL

General methods — Solutions were evaporated below 40° under diminished pressure. Melting points were determined with a Mel-Temp* melting-point apparatus (Laboratory Devices, Cambridge, Mass), and are uncorrected I r spectra were recorded with a Perkin–Elmer Model 257 grating i r spectrophotometer (The i r spectra of compounds **9**, **10**, **11**, and **13** have been published[29]) N m r spectra were recorded, at 60 MHz and ~40°, with a Varian A-60 n m r. spectrometer, for solutions (15–20%) in chloroform-*d* containing tetramethylsilane (τ = 10 00) as the internal

*Certain commercial products and instruments are identified in this paper in order to specify adequately the experimental procedure In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the product or equipment identified is necessarily the best available for the purpose

standard Each spectrum was first recorded at a sweep width of 500 Hz, then, the part of the spectrum containing the signals due to the ring protons was recorded at a sweep width of 100 Hz, and was calibrated from several of the sharpest lines in the same part of the former spectrum Chemical shifts are given on the τ-scale; the first-order J values given in Table I (p 561) were measured from the spectra of 100-Hz sweep-width, and the measurements are considered accurate to within ±0 1 Hz Unless otherwise stated, t l c was performed with Silica Gel G (E Merck, Darmstadt, Germany), activated at 110°, as the adsorbent and 2 1 ethyl acetate–pentane as the developer, and indication of zones was effected with sulfuric acid Microanalyses were made by W P. Schmidt

Isomerization of D-*xylose* (**1**) *or* D-*arabinose* (**2**) — In a one-necked (standard taper), 1-liter, round-bottomed flask were placed a Teflon-coated, egg-shaped, magnetic stirring-bar and finely powdered, anhydrous D-xylose or D-arabinose (150 g, 1 mole) Anhydrous pyridine (750 ml) was added, and the flask was quickly attached to a reflux condenser (Teflon sleeve and Drierite tube) and placed in a glycerol bath at 25° The bath was heated, and the suspension was magnetically stirred, after 30 min, all of the sugar had dissolved (bath temp 85°). The solution was then boiled, with stirring, for 4 5 h under reflux (bath temp 130°), cooled in ice to room temperature, and evaporated to a thick syrup under diminished pressure Several 100-ml portions of water were added and evaporated off, until the syrup had no odor of pyridine The dark-brown syrup was dissolved in distilled water (250 ml), and the solution was transferred to a 750-ml Erlenmeyer flask and made to 500 ml with water Activated carbon (6 g) was added, and the suspension was heated to 85°, with stirring, and filtered (sintered-glass funnel) The pale-yellow filtrate was evaporated under diminished pressure to a thick syrup which was freed of as much water as possible at 40°/0 3 torr, giving a yellowish mass of crystals and syrup (wt ~161 5 g) Absolute ethanol (300 ml) was added, and the suspension was boiled under reflux, the syrup and part of the crystals dissolved The suspension was cooled to room temperature, and filtered (rubber dam), and the crystals were washed with five 10-ml portions of absolute ethanol, and dried.

For the reaction of D-xylose (**1**), the first crop of recovered **1** weighed 77.8 g, and further crops (7 5 g) of **1** were obtained by evaporation of the mother liquor to a syrup and re-treatment with absolute ethanol (2 ml/g), total **1** recovered, 85 3 g (57%) The filtrate and washings were evaporated to a syrup which was dried at 35°/0 05 torr, wt 64 7 g

For the reaction of D-arabinose (**2**), the yield of crystalline **2** recovered was 98.0 g (65%), evaporation of the mother liquor gave a syrup, wt 50 9 g

Isolation of pentuloses — A solution of the syrupy mixture of pentoses in 1 liter of 3:1 (v/v) propyl alcohol–water was freed of aldopentoses by passage through a column (44 × 5 cm) of Amberlite IRA-400 (HSO_3^-) ion-exchange resin[17,18] The pentuloses were eluted with the same solvent (8 liters) at a flow rate of 5 ml/min. The effluent was collected in one portion and evaporated to a yellow syrup which was dissolved in water (200 ml), the solution was treated with decolorizing carbon,

and filtered, the colorless filtrate was evaporated to dryness, and the resulting syrup was dried at 32°/0.04 torr. The wt. of pentuloses from D-xylose (**1**) was 31 8 g; the wt from D-arabinose (**2**) was 29.4 g

Condensation with acetone — A solution of the syrupy mixture of pentuloses in water was transferred to a 1-liter, Pyrex bottle, and evaporated to a syrup which was dried to constant weight over phosphorus pentaoxide in a vacuum desiccator at 25°/0.1 torr Anhydrous copper(II) sulfate (40 g), reagent-grade acetone (400 ml), and concentrated sulfuric acid (0 4 ml) were added, and the mixture was shaken vigorously for 48 h at 25°. The suspension was filtered with suction, the solids were washed with acetone, and the filtrate and washings were combined and shaken with dry calcium hydroxide powder (15 g) for 1 h at 25°. The suspension was filtered through a layer of calcium hydroxide, and the filtrate was evaporated to a syrup which was dried at 32°/0 4 torr The wt of crude product from D-xylose (**1**) was 38 8 g, the wt from D-arabinose (**2**) was 41 0 g The syrupy mixture was treated with 400 ml of anhydrous ether, insoluble material was removed by filtration (fluted filter), and the colorless filtrate was evaporated to dryness The acetals from D-xylose (**1**) crystallized to a solid mass, wt 36 6 g, those from D-arabinose (**2**) constituted a syrup, wt 40 0 g

*Isolation of 2,3-O-isopropylidene-β-*D-threo*-pentulofuranose* (**13**) *from the pentulose acetals, by extraction — A Acetals from* D-*xylose* (**1**) The mixture of acetals (36 6 g) was dissolved in 500 ml of anhydrous ether, and the solution was extracted with five 20-ml portions of water, dried (anhydrous sodium sulfate), and evaporated to dryness, affording a mobile syrup (12 2 g) that consisted mainly of diacetals

The aqueous extracts were combined, and evaporated to a syrup which was dried at 32°/0 1 torr and then dissolved in 400 ml of anhydrous ether, the solution was dried (anhydrous sodium sulfate) and evaporated to dryness, affording a mass of colorless crystals of **13** (23 g) that was recrystallized as described in the next section

B Acetals from D-*arabinose* (**2**) The mixture of acetals (40 0 g) was processed in the same way The aqueous extracts were combined, and extracted once with ether (25 ml) The two ether layers were combined, washed with 25 ml of water, dried (anhydrous sodium sulfate), and evaporated, yielding crude **14** as a syrup (31 g) which had n_D^{20} 1 4473 and $[\alpha]_D^{25}$ −70 9° (*c* 0 5, acetone)

The aqueous extracts were treated with 2 g of activated carbon at 75°, the suspension was filtered (sintered glass) and the filtrate was processed as already described, affording a syrup (8 0 g) that was crystallized by dissolving it in anhydrous ether (6 0 ml/g), adding pentane to faint opalescence, nucleating with authentic **13**, and keeping the solution overnight in a freezer After one recrystallization, compound **13** was obtained as colorless crystals (7.3 g), m p 73°, n_D^{22} 1 4677 (superfused), $[\alpha]_D^{25}$ +1 5° (*c* 3, acetone) {lit [1] m p 70–71°, n_D^{22} 1 4671 (superfused), $[\alpha]_D^{25}$ +1 6° (*c* 4, acetone)}, ν_{max}^{KBr} 3380 (broad), 2995 and 2985 (doublet), 2975, 2950, 2920, 2885. 2865, 1460, 1440, 1430, 1385 and 1372 (doublet), 1350, 1338, 1322, 1258, 1238, 1210, 1178, 1130, 1105, 1080 and 1068 (doublet), 1028, 982, 962, 916, 876 and 866 (doublet), 848, 814, 778, and 700 cm^{-1} (see ref 29), n.m r. data. sharp singlets at τ 8 52 and

τ 8 66 (3-proton singlets, CMe_2) and τ 6 19 (OH) (confirmed by deuterium exchange), all other protons appeared as an unresolved multiplet between τ 5 57 and 6 16

Isolation of 2,3-O-isopropylidene-β-D-threo-pentulofuranose (**13**) *and of 1,2 3,4-di-O-isopropylidene-D-erythro-pentulofuranose* (**14**) *from the pentulose acetals from D-arabinose, by distillation* — A sample (64 7 g) of the syrupy mixture of pentulose acetals was fractionally distilled 1,2 3,4-Di-*O*-isopropylidene-D-*erythro*-pentulofuranose (**14**) was obtained as a colorless, mobile liquid (26.0 g), b p 61–72°/0.5 torr, n_D^{22} 1 4420, $[\alpha]_D^{25}$ −104 4° (*c* 1, acetone) The compound crystallized on being cooled in Dry Ice–acetone and kept in a freezer The i r spectrum showed ν_{max}^{film} 2985, 2935, 2880, 1455, 1380 and 1370 (doublet), 1295, 1265, 1245, 1210, 1155, 1095, 1070, 1044, 1038, 1012, 972, 942, 905, 872, 858, 816, 790, and 758 cm^{-1}; the n m r data are listed in Table I

After collection of several intermediate fractions, crude 2,3-*O*-isopropylidene-β-D-*threo*-pentulofuranose (**13**) was obtained as a viscous syrup (8 3 g), b p. 110–142°/0 2 torr, n_D^{25} 1 4676, $[\alpha]_D^{25}$ −23 9° (*c* 1, acetone) This syrup (8 3 g) was partioned between ether (20 ml) and water (20 ml), the aqueous layer was washed with ether (10 ml), and evaporated to a syrup which was dried at 0 1 torr over phosphorus pentaoxide, affording a crystalline mass It was recrystallized twice from ether–pentane, to give **13** as colorless crystals (7 5 g), m p 74°, n_D^{22} 1 4669 (superfused), and $[\alpha]_D^{25}$ +1 6° (*c* 1, acetone)

D-erythro-*Pentulose* (p-*tolylsulfonyl*)*hydrazone* — The procedure of Zinner and Rehpenning[18] was followed From 1 5 g of pure D-*erythro*-pentulose, prepared by hydrolysis of **14** (2 3 g) with 100 mM oxalic acid for 2 h at 65°, was obtained 512 mg (16%) of recrystallized hydrazone as microcrystals, m p 164° (dec), $[\alpha]_D^{25}$ +6 62 (15 min)→ −4 68° (48 h, *c* 0 8, pyridine), lit [18] m p 164° (dec), $[\alpha]_D^{21}$ +17 7 (10 min)→ +3 7° (24 h; *c* 1 0, pyridine) The i r spectrum showed ν_{max}^{KBr} 3460, 3310, 3145, 3050, 3010, 2960, 2930, 2900, 2860, 1860, 1595, 1510, 1450, 1430, 1395, 1380, 1350, 1320, 1310, 1292, 1266, 1240, 1188, 1155, 1082, 1032, 1022, 990, 968, 918, 882, 848, 820, 795, 758, and 718 cm^{-1}

Anal Calc for $C_{12}H_{18}N_2O_6S$ C, 45 3; H, 5 7; N, 8 8 Found C, 45 5, H, 5 7, N, 8 8

3,4-O-Isopropylidene-D-erythro-pentulofuranose (**17**) — A solution of 11 01 g (47 9 mmoles) of compound **14** in 50 ml of 4.1 (v/v) acetic acid–water was heated (under a reflux condenser) for 2 h at 65°, cooled, and lyophilized to give a syrup, three 100-ml portions of benzene were added and removed by lyophilization, to yield a syrup (9.35 g) practically free from acetic acid The syrup was shaken with three 50-ml portions of ether, and the supernatant solution was decanted each time; the residue was freed of solvent under vacuum, to give 2 33 g (32%, calc as **6**) of syrupy D-*erythro*-pentulose

The ether extracts were combined, dried (anhydrous sodium sulfate), and evaporated to a syrup (6 85 g), this was dissolved in water (50 ml), and the solution was extracted with three 25-ml portions of ether The extracts were combined, dried (anhydrous sodium sulfate), and evaporated to a syrup (3 16 g; 29%, calc as **14**)

The aqueous layer was evaporated to a syrup, which was dried, over phosphorus pentaoxide, at 25°/0 1 torr, wt 3 49 g (39%, calc as **17**) This syrup was distilled at 106°/0 25 torr, yielding syrupy 3,4-*O*-isopropylidene-D-*erythro*-pentulofuranose (**17**), wt. 1.70 g (19%, based on **14**, 26%, based on **14** and **14** recovered), $[\alpha]_D^{25}$ −63 0° (*c* 0 9, acetone), $[\alpha]_D^{25}$ −60 7 (10 min) → −60.9° (24 h, *c* 3.2, water) The syrup was dissolved in anhydrous ether (2 0 ml/g), and the solution was kept overnight in a freezer, whereupon crystallization occurred, affording 154 mg (9 2% of the syrup) of **17α**, m p 70–71°, $[\alpha]_D^{25}$ −5 4° (*c* 0 7, acetone), $[\alpha]_D^{25}$ −17 7 (10 min) → −60 9° (24 h, *c* 1 3, water), ν_{max}^{KBr} 3380, 3300, 2985, 2960, 2945, 2885, 1450, 1420, 1380 and 1370 (doublet), 1340, 1320, 1280, 1260, 1230, 1210, 1192, 1168, 1120, 1098, 1062, 1050, 1006, 976, 930, 904, 892, 862, 826, 780, and 725 cm^{-1}, the n m r data are given in Table I

Anal Calc for $C_8H_{14}O_5$ C, 50 5; H, 7 4 Found C, 50 8, H, 7 5

2,3-O-Isopropylidene-D-erythrono-1,4-lactone (**18**) — A solution of syrupy **17** (914 mg, 4 81 mmoles) in water (10 ml) was stirred in an ice bath, an ice-cold solution of sodium metaperiodate (1 030 g, 4 81 mmoles) in water (10 ml) was added in one portion, and the solution was stirred for 4 h at 0° The ice-cold solution was then extracted with four 10-ml portions of chloroform, and the extracts were combined, dried (anhydrous sodium sulfate), and evaporated to a crystalline mass (520 mg, 69%) Two recrystallizations from cold, dry ether afforded colorless crystals of **18**, m p 69°, $[\alpha]_D^{25}$ −111 9° (*c* 2 2, acetone), $[\alpha]_D^{22}$ −116 2° (initial, *c* 2, water) {lit [10] m p 65–67 5°, $[\alpha]_D^{22}$ −116° (initial, *c* 1, water)}, ν_{max}^{KBr} 2990, 2980, 2940, 1762, 1468, 1388 and 1376 (doublet), 1320, 1268, 1248, 1234, 1228, 1220, 1195, 1162, 1110, 1070, 1002, 974, 966, 930, 905, 860, 810, 790, 745, and 700 cm^{-1}, n m r data (in acetone-d_6 at 60 MHz) τ 5 16 (doublet, H-2), τ 5 80 (octet, H-3), τ 5 28 (quartet, H-4), τ 5 55 (quartet, H-4'), $J_{2,3}$ 5 9 Hz, $J_{3,4}$ 3 2 Hz, $J_{3,4'}$ 1 2 Hz, and $J_{4,4'}$ 11 0 Hz

Anomers of 1,2-di-O-acetyl-3,4-O-isopropylidene-D-erythro-pentulofuranose (**19**) — Syrupy **17**, or crystalline **17α**, (85 mg) was dissolved in an ice-cold solution of acetic anhydride (0 2 ml) in pyridine (2 ml) The solution was kept for 2 h at 0°, and for 20 h at room temperature, and then evaporated to a syrup; this was dissolved in chloroform, and the solution was washed successively with saturated, aqueous potassium hydrogen sulfate solution, water, saturated aqueous sodium hydrogen carbonate solution, and water, dried (anhydrous sodium sulfate), and evaporated to a syrup, wt 106 mg (87%) T l c of the products obtained (from either syrupy or crystalline **17**), with 7 3 (v/v) benzene–ethyl acetate as the developer, showed two spots, at R_F 0 43 and 0 65 The spots were of approximately equal intensity for the acetates from syrupy **17**, but the faster-moving product preponderated in the mixture from crystalline **17α** Neither anomer of **19** was obtained crystalline.

Anomers of methyl D-erythro-*pentulofuranoside* (**20**) — A solution of 5 2 g (34 7 mmoles) of **6** in 300 ml of 1% hydrogen chloride in methanol was kept for 1 h at room temperature, silver carbonate (25 g, 91 mmoles) was added, and the suspension was stirred for 2 h, and filtered. The filtrate was evaporated to dryness, giving a light-yellow syrup (5 3 g, 100%), $[\alpha]_D^{25}$ −62 5° (*c* 1, pyridine), no carbonyl

TABLE I

CHEMICAL SHIFTS AND FIRST-ORDER COUPLING CONSTANTS FOR SOME DERIVATIVES OF D *erythro* PENTULOSE

Compound No	*Chemical shifts*[a], τ *H-1*[b]	*H-1′*	*H-3*	*H-4*	*H-5*[b]	*H-5′*	*Me*	*OH*	*OMe*	*OAc*	*OBz*
14	5 75d[c]	6 02d	5 52d	5 21o	6 04q	6 24q	8 57s 8 60s 8 62s 8 70s				
17α	6 16s		5 42d	5.07o	5 87q	6 0⁴q	8 52s 8 68s	6 29s[d]			
21α	5 73s		4 75d	4 64o	5 64q	6 08q			6 63s	7 88s 7 92s 7 94s	
21β	5 63d	5 89d	4 77d	4 62o	4 82q	6 20q			6 78s	7 92s 7 97(2)s	
22α	5 45s		4 40d	4 27o	5 52q	5 90q			6 50s		2 13m 2.70(2)m
22β	5 08d	5 56d	4 25d	4 15o	5 53q	5 88q			6 64s		2.16m 2.74(2)m

Table I (*continued*)

Compound No	*First-order coupling-constants (Hz)* $J_{1,1'}$	$J_{3,4}$	$J_{4,5}$	$J_{4,5'}$	$J_{5,5'}$
14	9 6	5.4	5 4	1 6	9 6
17α		6 1	5 8	2 0	9 6
21α		7 1	12 6	5 1	10 7
21β	12 0	5 0	12 4	6.3	9 5
22α		5 2	10 8	2 6	10 8
22β	12 4	9 0	10 1	3 9	9 5

[a]With tetramethylsilane (τ 10 00) as internal standard [b]The proton on C-1 or C-5 giving the higher-field signal is arbitrarily designated H-1′ or H-5′, respectively [c]Signal multiplicities are indicated by d (doublet), m (multiplet), o (octet), q (quartet), or s (singlet) [d]Confirmed by deuterium exchange

band in the i r spectrum T l c on Kieselguhr G (E Merck, Darmstadt, Germany, activated at 110°), with 16 6 3 (v/v) ethyl acetate–isopropyl alcohol–water as the developer, showed two spots, of R_F 0 44 and 0 68.

Anomers of methyl 1,3,4-tri-O-*acetyl*-D-erythro-*pentulofuranoside* (**21**) — To an ice-cold solution of 1 8 g (11 mmoles) of **20** in 10 ml of pyridine was added 6 ml of acetic anhydride, and the solution was kept overnight at room temperature Ice (about 20 g) was added, and the resulting solution was extracted with chloroform The extract was washed successively with saturated aqueous potassium hydrogen sulfate solution, water, and saturated aqueous sodium hydrogen carbonate solution, dried (anhydrous sodium sulfate), and evaporated to a syrup, wt 2 3 g (85%). A solution of this syrup (2 3 g) in 5 ml of methanol was applied in a streak (6–8 mm wide) to five preparative, thin-layer plates (18 cm × 20 cm × 600 μm) of Kieselguhr G (E Merck, Darmstadt, Germany, 20 g per plate), which were developed with 7 3 (v/v) benzene–ethyl acetate Zones were detected by the technique of Horton and Tsuchiya[30], and were scraped from the plates without great regard for high recovery (in order to obtain each anomer pure) For each plate, two zones were thus obtained; similar zones were combined, and the material was separated from the adsorbent by extraction with methanol In this way, the α-D anomer (**21α**) was isolated as a syrup (0 7 g), R_F 0 35, $[\alpha]_D^{25}$ +24 0° (*c* 0 6, acetone); the n.m r data are given in Table I

Anal Calc for $C_{12}H_{18}O_8$ C, 49 7, H, 6 3. Found. C, 50 0, H, 6 6

The β-D anomer (**21β**) was also isolated as a syrup (0 4 g), R_F 0 50, $[\alpha]_D^{25}$ −56 1° (*c* 1, acetone), the n m r data are given in Table I

Anal Calc for $C_{12}H_{18}O_8$ C, 49 7, H, 6 3 Found C, 50 0, H, 6 5

Anomers of methyl 1,3,4-tri-O-*benzoyl*-D-erythro-*pentulofuranoside* (**22**) — A solution of 5 3 g (32 3 mmoles) of **20** in 40 ml of dry pyridine was cooled in an ice–salt bath, and benzoyl chloride (12 4 ml, 107 mmoles) was added with vigorous stirring The solution was stirred for 15 min in the ice–salt bath, kept for 1 h at room temperature, and recooled in ice–salt. Moist pyridine (50 ml containing 10 drops of water) was added, and the solution was stirred, with cooling, for 40 min Saturated aqueous sodium hydrogen carbonate solution (20 ml) was added, and stirring and cooling were continued for 30 min A further 20 ml of saturated aqueous sodium hydrogen carbonate solution was added, the resulting solution was evaporated to dryness, and the mixture was partitioned between 200 ml of chloroform and 200 ml of water The layers were separated, and the aqueous layer was extracted with chloroform and discarded The chloroform extracts were combined, washed successively with saturated aqueous potassium hydrogen sulfate solution, water, saturated sodium hydrogen carbonate solution, and water, dried (anhydrous sodium sulfate), and evaporated to a syrup (14 2 g, 91%)

A portion (1.5 g) of this syrup was dissolved in 4 ml of chloroform, and subjected to preparative t l c as described for **21**, except that the developer was 19.1 (v/v) benzene–ether One zone afforded crystalline **22α** (0 3 g), R_F 0 45; after one recrystallization from 2 1 (v/v) ethanol–pentane, it had m p 98°, $[\alpha]_D^{25}$ −62 2°

(c 0 6, chloroform); ν_{max}^{KBr} 3075, 3055, 2985, 2965, 2945, 2890, 2840, 1720, 1600, 1580, 1490, 1450, 1370, 1352, 1315, 1265, 1168, 1147, 1120, 1108, 1088, 1069, 1052, 1025, 980, 952, 882, 854, 812, 808, 790, 744, 720, 710, 688, 674, and 660 cm^{-1}, the n.m r data are given in Table I

Anal Calc for $C_{27}H_{24}O_8$ C, 68 1, H, 5 1. Found: C, 68 0; H, 4 9

The second zone gave syrupy **22β** (0 2 g), R_F 0 60, $[\alpha]_D^{25}$ −111 3° (c 0 7, chloroform), the n m r data are given in Table I

Anal Calc for $C_{27}H_{24}O_8$ C, 68 1; H, 5 1 Found C, 68 0; H, 5 1

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CONFORMATIONAL STUDIES ON PYRANOID SUGAR DERIVATIVES THE CONFORMATIONAL EQUILIBRIA OF β-D-RIBOPYRANOSE TETRAACETATE AND β-D-XYLOPYRANOSE TETRAACETATE*

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ABSTRACT

β-D-Ribopyranose tetraacetate (**1**) has been shown to exist, in acetone-d_6 at room temperature, as a 9 11 mixture of the *1C* (D) and *C1* (D) conformers in rapid equilibrium The rate of chair–chair interconversion at −60° is 130 times per second. At −84°, a temperature at which n m r spectral signals of the two separate conformers can be observed, the ratio of the *1C* (D) to *C1* (D) conformers is 2 1 For β-D-xylopyranose tetraacetate (**2**), in acetone-d_6 at room temperature, the all-axial, *1C* (D) conformer exists in equilibrium with the all-equatorial, *C1* (D) conformer to the extent of 1 part in 5 At −84°, the *1C* (D) conformer was not detected

INTRODUCTION

Conformational analysis of pyranoid sugars and their derivatives may be expected to follow the broad principles established with cyclohexane derivatives[3], but there are major points of difference[4,5] The symmetry of the ring is decreased by introduction of an oxygen atom[6], and the latter exerts a stereoelectronic influence on the conformation that is different from that of a methylene group The availability of well-characterized, complete configurational series of various sugar derivatives makes the sugars particularly attractive models for the study of interactions between substituents in polysubstituted systems, and of interactions involving a hetero atom in the ring systems

Pyranoid sugars and their derivatives may be formulated in two, energetically nonequivalent, chairlike conformations, interconversion between which involves an energy barrier corresponding to a conformer having five ring-atoms in one plane,

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and also in a "flexible cycle" of skew forms[7] interconvertible through energy barriers corresponding to the boat forms At conformational equilibrium, the various conformers will be populated to extents depending on their relative free-energies, according to the classical thermodynamic distribution; the concentrations of those conformers corresponding to the energy barriers will be infinitely small.

The relative, free-energy content of a particular conformer is determined by a combination of (*a*) steric interactions (attractive and repulsive van der Waals interactions), (*b*) bond-torsional (Pitzer) strain, (*c*) bond-angle (Baeyer) strain, (*d*) electronic factors involving interaction of dipoles, and (*e*) the effects of solvation and hydrogen bonding Experimental evidence, mainly from n m r spectroscopy[8-12], has suggested that most pyranoid sugars and their monocyclic derivatives exhibit a high degree of conformational homogeneity in one of the two chairlike conformations, convincing evidence for nonchair conformers as favored forms has been presented only in the case of systems having other rings fused to the pyranose ring[13]

The apparent conformational homogeneity of most pyranoid sugar derivatives may be the consequence of rapid conformational interconversion, so that a time-averaged spectrum is observed, but only the favored conformer makes a substantial contribution Alternatively, interconversion may be slow on the n m r time-scale, so that superimposed spectra of the two chair conformers are obtained, but the proportion of the minor conformer is so small that its signals are lost in the background noise of the spectrum The present work[1,2] provides direct evidence that the energy barrier for chair–chair interconversion in a pyranoid sugar derivative is small enough for a time-averaged spectrum to be observed at room temperature, indirect evidence for this conclusion can be inferred from the fact that certain pyranoid sugar derivatives[10,14-17] give n m r spectral parameters intermediate between those to be expected[8-12] for the separate chair conformers

Methods for measuring conformational equilibria — The n m r spectroscopic methods that have been used for determining conformational equilibria, as in six-membered ring-systems, have involved observing the signal of a particular proton (or protons) in the time-averaged environment and relating this signal to the signals observed (or anticipated for) each chair conformer alone From these data, the mole fractions (N_e and N_a) of the conformers having that particular proton equatorial and axial, respectively, have been determined The standard free-energy difference $\Delta G°$, and equilibrium constant K for the interconversion H-*eq* $\rightleftarrows$ H-*ax*, can then be calculated from the relationship

$$\Delta G° = -RT\ln K = -RT\ln \frac{N_a}{N_e}.$$

Four different methods for determining N_e and N_a have been used

(*1*) *Method of averaging of spin coupling*[18,19] The values of N_e and N_a are determined from the equation

$$J_{obs} = N_e J_e + N_a J_a,$$

which relates a particular spin-coupling (J_{obs}) of the proton in question, in the time-averaged spectrum, with that (J_e) when the proton is exclusively equatorial and that (J_a) when it is exclusively axial For this method to give useful results, the values of J_e and J_a must differ substantially, and these values must be measured directly, as by low-temperature n m r spectroscopy, or reliable estimates of their magnitudes, made from suitable reference compounds, must be available

(*2*) *Method of averaging of chemical shifts*[20] In this method, the values of N_e and N_a are determined from the relationship, namely,

$$\nu_{obs} = N_e \nu_e + N_a \nu_a,$$

between (*a*) the chemical shift (ν_{obs}) of the proton in the time-averaged spectrum and (*b*) the chemical shifts (ν_e and ν_a) when the proton is in the wholly equatorial or wholly axial environment The latter values are determined directly by measurement of the spectrum at a temperature sufficiently low that the signals of the two conformers are observed independently because interconversion is slow on the n.m r. time-scale

(*3*) *Direct integration below the temperature of conformational "freeze-out"* By this method[21], the mole fractions N_e and N_a are measured directly by integration of the spectrum at a temperature at which signals of the separate conformers are observed

(*4*) *Reference to chemical shifts in conformationally biased systems* This method, introduced by Eliel[22], resembles method (*2*), except that ν_e and ν_a are not measured directly but are estimated by reference to related compounds that have a substituent (such as *tert*-butyl) that causes one chair conformer or the other to be overwhelmingly favored

None of these methods can be expected to provide meaningful values for $\Delta G°$ and K, even under optimum conditions, unless the proportion of the minor conformer is at least 5–10%, because proportions less than 5% would be difficult to detect and measure by present instrumental techniques

Rate of conformational inversion — The frequency with which one chair conformer undergoes ring-flip to the alternative chair conformer may be determined by n m r spectroscopy, provided that (*a*) spectral features characteristic of the separate conformers can be observed at a temperature sufficiently low for interconversion to be slow on the n m r time-scale, and (*b*) measurements can be made at progressively higher temperatures until a fully time-averaged spectrum of the conformers is observed Considering the resonance of an individual proton, whose chemical shift is ν_e when it is in the equatorial orientation and ν_a when it is in the axial orientation, it is possible in principle to determine $\nu_a - \nu_e$ by measurement of the spectrum at a temperature low enough for interconversion to be slow and the separation of the two signals to be at the maximum As the temperature is raised and interconversion becomes more rapid, the separate signals broaden, coalesce, and eventually become a single, sharp signal having a chemical shift intermediate between ν_a and ν_e The rate of interconversion k at T_c, the temperature of coalescence

(when the two broadened signals just become a single, broad signal) is given by the equation of Gutowsky and Holm[23]

$$k = \frac{\sqrt{2}}{2}\pi|\nu_a - \nu_e|,$$

where k and $(\nu_a - \nu_e)$ are expressed in Hz From these values, the free energy of activation $\Delta G^{\ddagger}$ for the interconversion can be calculated from the Eyring equation

$$k = \frac{\kappa k_B T_c}{h} e^{-\Delta G^{\ddagger}/RT_c},$$

where k_B is the Boltzmann constant, κ is the transmission coefficient, and h is the Planck constant

Conformational equilibria and rates of conformational inversion in pyranoid sugar derivatives — In order to detect and measure a conformational equilibrium and rate of ring-flip in a pyranoid sugar derivative, it is necessary to have (*i*) a reasonably concentrated (5–10%) solution that can be observed in the n m r spectrometer over a wide temperature-range without crystallization of the solute, boiling of the solvent, freezing of the solution, or development in the solution of high viscosity that would lead to excessive line-broadening and loss of necessary spectral detail, (*ii*) a compound in which the proportion of the less favored conformer is adequate for detection at the temperature of "conformational freeze-out", and (*iii*) a spectrum that is amenable to analysis, preferably on a first-order basis, so that the signal of one or more protons can be observed and measured accurately For all of these reasons, it is desirable to use a spectrometer of high resolving power, operating at the highest field-strength attainable The temperature required for "conformational freeze-out" may be inconveniently low for a spectrometer operating at 60 or 100 MHz, and the use of a 220-MHz spectrometer may be especially advantageous for achieving satisfactory spectral dispersion and for bringing the temperature for "conformational freeze-out" into a more accessible range

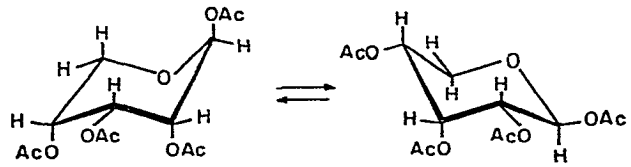
In the present work, the conformational equilibria of β-D-ribopyranose tetraacetate (**1**) and β-D-xylopyranose tetraacetate (**2**) were studied by method *1* (averaging of spin coupling) In the case of compound **1**, it was also possible to apply methods *2* and *3*, and thus to make an intercomparison of the three methods The last two methods were inapplicable with **2**, because, at the temperature range at which "conformational freeze-out" might be expected, the *C1* (D) conformer was too strongly favored to permit observation of signals of the *1C* (D) conformer The rate of conformational inversion of **1** was measured

RESULTS AND DISCUSSION

β-D-Ribopyranose tetraacetate (**1**)

The direct observation, by low-temperature n m r spectroscopy, of signals attributable to the separate conformers of **1** establishes that these conformers are

in rapid equilibrium at room temperature, the spectrum observed above −30° is a time-averaged pattern



1C (D) *C1* (D)

1

Analysis of spectra — The 220-MHz n m r spectrum of **1** in acetone-d_6 at room temperature (Fig 1, lowest curve) was completely first-order, and analysis[11,12] gave the following first-order couplings· $J_{1\,2}$ 4 8, $J_{2\,3}$ 3 0, $J_{3\,4}$ 3 6, $J_{4\,5}$ 3 2, $J_{4\,5'}$ 5 6, $J_{3\,5}$ ~0 5, and $J_{5\,5}$ 12 0 Hz These values are intermediate between those predicted[9,11,12,15] for **1** in the *C1* (D) conformation ($J_{1\,2}$ 8–10 Hz) and those predicted for **1** in the *1C* (D) conformation ($J_{1,2}$ 1–2 Hz) The temperature of the sample was

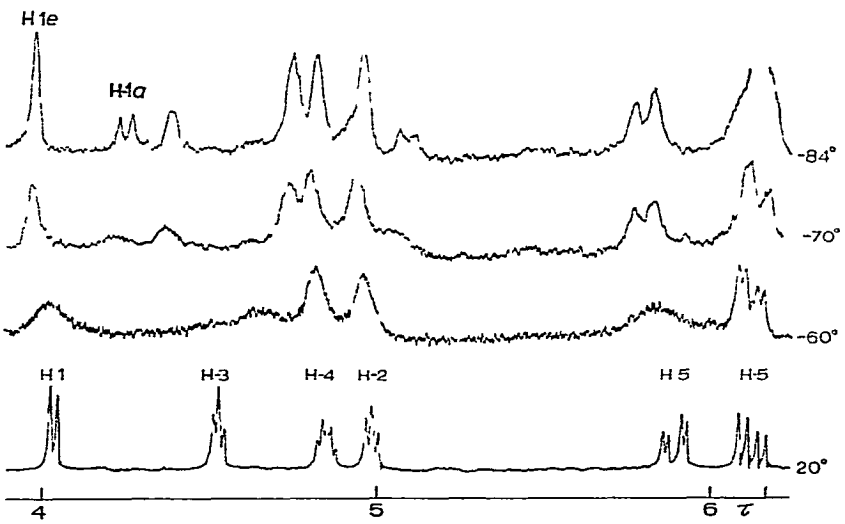


Fig 1 The low-field portion of the 220-MHz n m r spectrum of β-D-ribopyranose tetraacetate (**1**) in acetone-d_6 at 20, −60, −70, and −84°

lowered in stages, and the spectrum showed progressive changes At −32°, slight line-broadening was evident, and, with decreasing temperature, the H-1, H-3, and H-5 signals collapsed to broad humps The shapes of the other signals broadened, and the appearance of the spectrum at −60° is shown in the second curve in Fig 1. New signals began to appear as the temperature was lowered further, and the appearance of the spectrum at −70° is shown in the third curve of Fig 1 The signals became sharper at still lower temperatures, and the spectrum observed at −84° (Fig 1, top curve) was not substantially different from that observed at −78°. The

TABLE I

CHEMICAL-SHIFT DATA FOR β-D-RIBOPYRANOSE TETRAACETATE (1) AND β-D-XYLOPYRANOSE TETRAACETATE (2) IN ACETONE-d_6 AT 100 MHz

Compound	*Temperature, degrees*	*Chemical shifts, τ*						*Acetyl signals*[a]
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-5'*	
1	+30	4 03	4 98	4 53	4 84	5 89	6 15	7 88, 7 93, 7 94, 7 96
	−40	4 02	4 96	4 55	4 81	5 85	6 14	7 84, 7 90, 7 91, 7.96
	−60	4 03	4 96	4 58	4 82	5 84	6 15	7 83, 7 90[b], 7 94
	−85							7 78[c], 7 81[c], 7 83[c], 7 96[c], 8 02[c]
2	+30	4 20	[d]	4 71	[d]	5 87	6 36	7 93, 7 98[e], 7 99
	−60	4 18	[f]	4 59	[f]	5 90	6 32	7 90, 7 97[e] 7 98
	−80	4 16	[g]	4 52	[g]	5 91	6 31	

[a]Unless otherwise indicated, acetyl-proton signals were approximately 3-proton singlets [b]6-Proton singlet [c]Signals of unequal intensities. [d]Multiplet near τ 5 06. [e]6-Proton singlet [f]Multiplet near τ 5 03 [g]Multiplet near τ 5 01

signals for the acetoxyl groups also showed changes, from a simple, 4-line pattern at 20° to a more complex one at −84° Details of chemical shifts are given in Table I

The observed data indicate that compound **1** in acetone-d_6 exists, at room temperature, as a mixture of conformers undergoing rapid interconversion, and that, at −84°, the interconversion is slow on the n m r time-scale so that signals of the separate conformers are seen Assuming that H-1 of either conformer gives its signal at a field-position lower than those of the other ring-protons, the broadened singlet at lowest field (τ 3.93) and the wide doublet at next higher field (τ 4 19) can be assigned to anomeric protons of separate conformers* The former signal can be assigned to H-1 of the *1C* (D) conformer, it shows only small coupling with H-2 ($J_{1e,2e} \sim 1$ Hz), in line[24] with the diequatorial arrangement of H-1 and H-2 in this conformer. The higher-field, anomeric-proton signal was assigned to H-1 of the *C1* (D) conformer; this axial proton is strongly coupled with the axial H-2 ($J_{1a,2a}$ 8 0 Hz) The relative magnitudes of the signals indicate that the *1C* (D) and *C1* (D) conformers are present in the ratio of 2 1, so that the equilibrium constant for the conformational inversion *1C* (D) ⇄ *C1* (D) is 0 5 at −84°, and the value of ΔG°_{-84} is ±0 26 +0 07 kcal mole^{-1}

The chemical shifts observed for the anomeric proton of each conformer accord with Lemieux's generalization[9 25] that axial protons usually resonate at higher field than equatorial protons By Lemieux's empirical rules[9] on the effect of configuration on chemical shift, the estimated value for the chemical shift of H-1*e* in **1** is τ 3 85 (observed, τ 3 93) and that for H-1*a* is τ 4 00 (observed, τ 4 19)

The remainder of the 220-MHz spectrum of **1** in acetone-d_6 at −84° showed, upfield from the H-1 signals, three signals whose intensities were similar to those of the H-1*e* signal These signals presumably arise from the other methine protons of the *1C* (D) conformer Two additional signals, of intensity equal to that of the H-1*a* signal, are observed and are presumably those of two of the methine protons of the *C1* (D) conformer, and the signal of the remaining methine proton of the *C1* (D) conformer appears as a broad resonance partially overlapped by one of the stronger signals

Conformational equilibrium at room temperature — Given that $J_{1a,2a}$ for the *C1* (D) conformer of **1** is 8 0 Hz, and assuming that $J_{1e\ 2e}$ for the *1C* (D) conformer is 1 0 Hz**, application of the method of averaging of spin coupling to the spectrum at 20°, where the observed $J_{1\ 2}$ is 4 8 Hz, gives, for the interconversion *1C* (D) ⇄ *C1* (D), an equilibrium constant K of 1 2 and a standard free-energy difference ΔG°_{20} of −0 11 ±0 09 kcal mole^{-1} This value differs from the one determined by integration, because the latter refers to the equilibrium at −84° and the equilibrium constant increases with rise of temperature

*In the 100-MHz spectrum of tri-*O*-benzoyl-β-D-ribopyranosyl cyanide, at −64° in chloroform-*d*, Coxon[15] has observed signals tentatively identified as those of H-1*e* and H-1*a* of the separate conformers

The spectrum of **1 at −84° was insufficiently well resolved to permit accurate measurement of $J_{1e,2e}$ From measurements on 19 examples in conformationally locked aldohexopyranose derivatives, Coxon[24] observed an average value of $J_{1e,2e}$ of 1 0 ±0 3 Hz

Application of the method of averaging of chemical shifts, by comparing the shifts of H-1*e* and H-1*a* at −84° with that of the time-averaged H-1 at 20°, would suggest that the equilibrium constant is 0 61 at 20°, corresponding to a ΔG°_{20} value of +0 30 ±0 09 kcal mole^{-1}. This estimate differs substantially from the equilibrium constant of 1 2 determined by the method of averaging of spin coupling The latter value is undoubtedly the more correct one The discrepancy probably results from variation of the chemical shifts of the H-1 resonances of the two conformers with temperature, leading to a spurious estimate (0 61) of K by the chemical-shift method Eliel and Martin, also, have observed a similar discrepancy in their studies on the conformational equilibria of cyclohexyl halides[26].

Rate of conformational inversion — The separation of the H-1*e* and H-1*a* signals of **1** at −84° is 58 5 Hz at 220 MHz From this result, it may be calculated[23] that, at the coalescence temperature (T_c) of −60°, the rate constant k for the chair–chair interconversion is 130 sec^{-1}. The energy barrier ($\Delta G^{\ddagger}$) for the interconversion was calculated from the Eyring equation[27] to be 10 0 ±0 2 kcal mole^{-1}, taking the transmission coefficient κ as 0 5 on the assumption (compare ref 28) that ring inversion proceeds by way of an intermediate in the flexible cycle This value is slightly less than $\Delta G^{\ddagger}$ for the conformational inversion of cyclohexane-d_{11}, for which Anet *et al* [28] gave 10 3 kcal mole^{-1} and Bovey *et al* [29] gave 10 2 kcal mole^{-1}, and is approximately equal to the value of 10 0 kcal mole^{-1} determined for tetrahydropyran from the data* of Gatti *et al* [30]

The changes observed at low temperature in the 220-MHz n.m r spectrum of **1** in acetone-d_6 could also be observed in spectra measured at 100 MHz and even at 60 MHz, but at lower temperatures The observed separation of the H-1*e* and H-1*a* signals below the temperature of "conformational freeze-out" (see Fig 2) was 25 5 Hz at 100 MHz, and 14 5 Hz at 60 MHz, these values are close to those calcu-

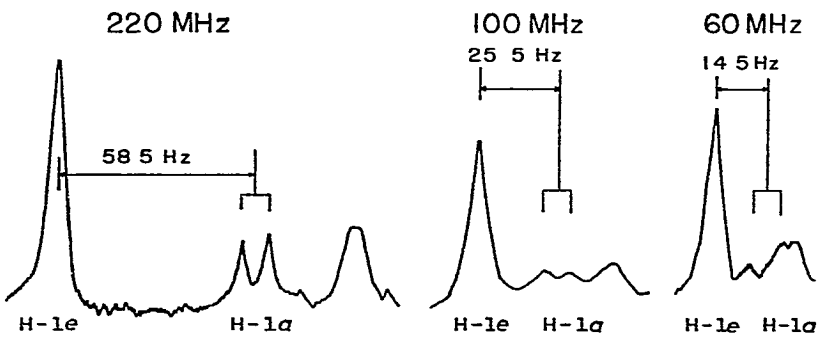


Fig 2. Appearance of signals of the anomeric protons of β-D-ribopyranose tetraacetate (**1**) in acetone-d_6 below the temperature of conformational freeze-out at 220, 100, and 60 MHz

*Each of these values is expressed on the basis that the transmission coefficient κ is 0 5 If κ is taken as unity, the $\Delta G^{\ddagger}$ values for cyclohexane-d_{11} are 10 6 kcal mole^{-1} (ref 28), and 10 5 kcal mole^{-1} (ref 29), for compound **1**, $\Delta G^{\ddagger}$ = 10 3 kcal mole^{-1}, and for tetrahydropyran[30], $\Delta G^{\ddagger}$ = 10 2 kcal mole^{-1}.

lated, from the separation (58.5 Hz) observed at 220 MHz, by simple proportion according to the spectrometer frequency As may be seen from Fig 2, it is more difficult to make exact measurements from the spectra at the lower field-strengths, because the spectral dispersion is less.

From the separation of the H-1*e* and H-1*a* signals at 100 and 60 MHz, application of the Gutowsky–Holm equation[23] leads to rate constants (k) of 57 and 33 sec^{-1}, respectively, at the respective coalescence temperatures (see Table II) The observed value for T_c was approximately −68° at 100 MHz, and approximately −73° at 60 MHz

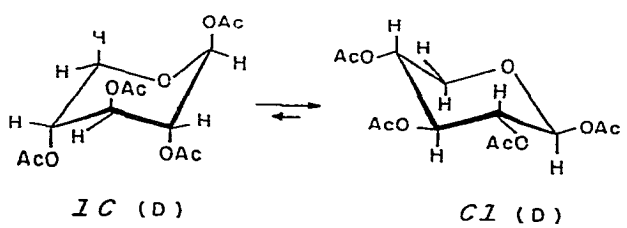
TABLE II

RATE OF CONFORMATIONAL INVERSION FOR β-D-RIBOPYRANOSE TETRAACETATE (1) IN ACETONE-d_6 AT VARIOUS TEMPERATURES

Spectrometer frequency, MHz	*Coalescence temperature* (T_c), *degrees*	*Rate of conformational inversion* (k) *in* sec^{-1} *at* T_c
220	−60	130 ±1
100	−68	57 ±1
60	−73	33 ±2

Effect of change of temperature on conformational equilibrium — As the temperature was lowered by su[illegible] from room temperature, it was observed at each spectrometer frequency that the magnitude of the observed $J_{1,2}$ value decreased regularly (4 8 Hz at 20°, 4 0 Hz at −32°), as would be expected from a shift in the equilibrium that would increasingly favor the *1C* (D) form (H-1 and H-2 diequatorial) at lower temperatures A quantitative relationship between $J_{1,2}$ (and, therefore, the equilibrium constant K by the method of averaging of spin coupling) and the temperature could not be obtained over an extended temperature-range, because of the line-broadening of the H-1 signal accompanying the "conformational freeze-out" However, in the case of the β-D-*xylo* analog, such a relationship could be determined

β-D-*Xylopyranose tetraacetate* (**2**)



1C (D) ⇌ *C1* (D)

2

Conformational equilibrium and its temperature dependence — The 100-MHz spectrum of **2** in acetone-d_6 at room temperature showed a $J_{1,2}$ value of approximately 6 9 Hz At −85°, this coupling increased to 8 1 Hz Further decrease of the

temperature caused no further increase in the value of $J_{1,2}$, and the value of 8.1 $\pm$0 1 Hz observed was taken to be $J_{1a,2a}$ of the pure *C1* (D) conformer below the temperature of "conformational freeze-out" No signal attributable to H-1*e* of the *1C* (D) conformer was detected, and it may be assumed that the latter conformer is present to the extent of $<5\%$ at $-85°$ The fact that the $J_{1,2}$ value at room temperature was substantially less than 8 1 Hz was taken to indicate the presence of an appreciable proportion of the *1C* (D) conformer, in rapid equilibrium with the *C1* (D) conformer, at that temperature Assuming a value of 1.0 $\pm$0 5 Hz for $J_{1e,2e}$ of the *1C* (D) conformer, by analogy with the *β*-D-*ribo* analog **1** and the data of Coxon[24] (see second footnote, p 571), the value of the equilibrium constant K for the interconversion *1C* (D) $\rightleftarrows$ *C1* (D) can be determined by the method of averaging of spin coupling

The value of K, as calculated from the observed $J_{1,2}$ value, was determined at various temperatures between $+68$ and $-65°$, and a least-squares plot of $\ln K$ *vs* $1/T$ (see Fig 3) gave a straight line having a positive slope The intercept of the

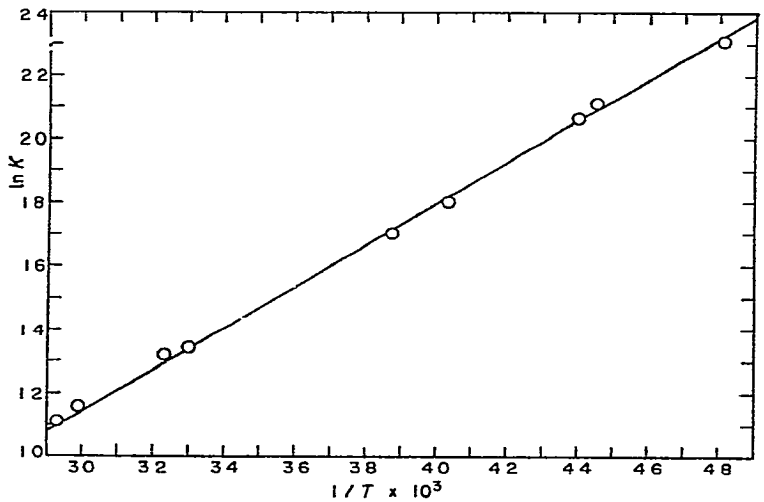


Fig 3 A plot $\ln K$ versus $1/T$°K for the conformational equilibrium of *β*-D-xylopyranose tetraacetate (**2**)

plot at $1/T = 0$ gives $\Delta S°/R$, and the slope gives[31] $-\Delta H°/R$ The standard enthalpy difference $\Delta H°$ thus determined is $-1\ 3 \pm 0\ 2$ kcal mole^{-1}, and the standard entropy difference $\Delta S°_{25}$ is $-1\ 6 \pm 0\ 3$ cal deg^{-1}mole^{-1} The standard free-energy differences at various temperatures, determined from the relationship $\Delta G_T = -RT\ln K_T$, are given in Table III This method provides values of ΔH° and $\Delta S°$ that are only approximate, but greater accuracy can be expected in the $\Delta G°$ values[31] At 25°, the *1C* (D) and *C1* (D) conformers are present in a 1 4 ratio ($\Delta G° = -0\ 82 \pm 0\ 13$ kcal mole^{-1}) in rapid equilibrium

As the temperature of the solution of **2** in acetone-d_6 was lowered, the H-1 signal shifted to lower field (see Table I) Had the true chemical shifts of H-1*a*

TABLE III

CONFORMATIONAL EQUILIBRIUM DATA FOR THE *1C* ⇌ *C1* CONFORMATIONAL INVERSION OF β-D-XYLO-PYRANOSE TETRAACETATE (2)

Temperature[a], *degrees C*	$J_{1,2}$, *Hz*[b]	*Equilibrium constant*[c], K = C1/1C	*Free-energy difference* (*ΔG°*), *kcal mole*$^{-1}$
−65	7 5	10 2	−0 97 ±0 16
−49	7 3	8 1	−0 94 ±0.16
−25	7 1	6 2	−0 90 ±0 15
−16	7 0	5 6	−0 88 ±0 14
+37	6 6	3 7	−0 81 ±0 13
+62	6 4	3 1	−0 76 ±0 13
+68	6 3	3 0	−0 75 ±0 13

[a]Within ±2° [b]Within ±0 1 Hz [c]Interpolated from a plot of ln*K versus* 1/*T*°K

[of the *C1* (D) conformer] and H-1*e* [of the *1C* (D) conformer] been independent of temperature, a small, upfield shift of the averaged H-1 signal should have been observed as the temperature was lowered, corresponding to an increase in the proportion of *C1* (D) conformer The fact that the change observed was in the opposite direction suggests that the chemical shifts of H-1*a* and H-1*e* are not independent of temperature, so that the method of averaging of chemical shifts[20,22] is quite unreliable[26] as a method for determining conformational equilibria

In general, for measurement of conformational equilibria for pyranoid sugars and their derivatives, the method of averaging of spin coupling appears satisfactory for those systems where the one conformer has a pair of vicinal protons in diaxial orientation, provided that (*a*) the signal of one of these protons can be observed clearly, and (*b*) at least 5–10% of the less-favored conformer is present The method of integration below the temperature of conformational freeze-out provides convincing evidence of conformational equilibria, but its use is restricted to those examples having substantial proportions of both conformers at low temperatures The *ΔG°* values observed at low temperature cannot be used in comparison with values at room temperature unless the temperature coefficient of the equilibrium constant is known

Factors influencing conformational stability — The observation that the *1C* (D) conformer of β-D-ribopyranose tetraacetate (**1**), having three acetoxyl groups axial, is almost as stable at room temperature as the *C1* (D) conformer, even though the latter has only one axial acetoxyl group, illustrates the strong influence of the "anomeric effect" in directing the conformational stability of pyranoid sugars having an acetoxyl group on the anomeric carbon atom In the case of the β-D-*xylo* analog **2**, the *1C* (D) form is present to the extent of 20% at room temperature, even though this conformer has all of its substituents axially attached. The anomeric effect of the acetoxyl group is less than that of a halogen atom; for example, tri-*O*-acetyl-β-D-xylopyranosyl chloride exists to the extent of at least 80% in the all-axial *1C* (D) conformation[1,32], and the corresponding β-D-*ribo* bromide analog appears[11]

to be exclusively *1C* (D) By comparison, the anomeric effect of an acetylthio group is less than that of an acetoxyl group, the 1-thio analog of **1** is[12] preponderantly *C1* (D)

The data presented herein for **1** and **2** all refer to solutions in acetone-d_6, a solvent selected because it gave satisfactory spectral dispersion and could be used at low temperature However, it may be expected that similar distributions of conformers, in rapid equilibrium at room temperature, will be found to exist in other solvents, because $J_{1\,2}$ values are observed of similar magnitude to those recorded for solutions in acetone-d_6 Some quantitative differences in the distribution of conformers can be anticipated, because of differences in dielectric constant of various solvents Forthcoming reports will be concerned with conformational and configurational equilibria, including the effects of various solvents, for various peracylated aldopyranoses and aldopyranosyl halides

EXPERIMENTAL

N m r spectral measurements — Spectra at 60 and 100 MHz were measured with Varian A-60A and HA-100 n m r spectrometers, respectively Spectra were measured for solutions containing ~30% of the sample in acetone-d_6, and tetramethylsilane (τ = 10 00) was used as the internal standard and to provide a lock signal Variable-temperature measurements at 60 and 100 MHz were made with a Varian V-4341/V-6057 variable-temperature accessory and a Varian V-6040 controller Calibration in the low-temperature range was effected with a methanol sample, and ethylene glycol was used for high-temperature calibration The temperatures recorded are considered accurate to ±2° Coupling constants for the plot of $\ln K$ versus $1/T$ (see Fig 3) were measured, as direct peak-spacings, from spectra recorded at a sweep-width of 100 Hz, and are considered accurate to within ±0 1 Hz, other coupling constants recorded are considered accurate to within ±0 3 Hz or better Chemical shifts are on the τ scale, and were taken from the chart recording and/or were measured electronically by using the "Diff 1" position on a Varian V-4354A Internal Reference NMR Stabilized Controller in conjunction with a Varian V-4315 Frequency Counter; values are considered accurate to within ±0 005 p p m

Spectra at 220 MHz were measured with a Varian HR-220 spectrometer equipped with a superconducting solenoid (compare F. A Nelson and H E Weaver, "High Resolution Superconducting Spectrometer", presented at the International Conference on Magnetic Resonance and Relaxation, XIVth Colloque Ampère, Ljubljana, Yugoslavia, September, 1966)

β-D-Ribopyranose tetraacetate (**1**) — D-Ribose (Pfanstiehl Laboratories, Inc, Waukegan, Illinois) was acetylated by the procedure of Levene and Tipson[33] to give **1**, m p 109–110° (lit [33] m p 110°), for the n m r spectral data, see Tables I and II, and Figs 1 and 2

β-D-Xylopyranose tetraacetate (**2**) — D-Xylose was acetylated by the procedure of Hudson and Johnson[34] to give **2**, m p 127–128° (lit [34] m p 128°), for the n m r. spectral data, see Table I

ACKNOWLEDGMENTS

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OXIDATION OF ALDITOLS BY MERCURY(II) ACETATE

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ABSTRACT

The secondary hydroxyl groups of D-arabinitol and xylitol were shown to undergo oxidation with a boiling, methanolic solution of mercury(II) acetate to give D-*threo*-pentulose, D-*threo*-3-pentulose, D-*erythro*-pentulose, and DL-*threo*-pentulose and *erythro*-3-pentulose, respectively. The formation of dihydroxyacetone and DL-glyceraldehyde from glycerol, and mainly glycolaldehyde from ethylene glycol, indicates that primary hydroxyl groups are also oxidised These results, as well as the formation of acetone upon treatment of isopropyl alcohol with mercury(II) acetate, indicate that cyclic intermediates are not involved in these oxidations

INTRODUCTION

There is considerable interest at present in the preparation of unsubstituted ketoses 2-Ketoses are usually prepared by transformation of the appropriate aldoses in pyridine (*e g*, D-tagatose[1], D-*erythro*-pentulose[2–4], D-*threo*-pentulose[5], and D-*glycero*-tetrulose[6]), by bacterial oxidation[7] of alditols, and by the diazomethane synthesis[8,9]

The preparation of 3-ketoses is more complicated than that of 2-ketoses, and can be achieved by oxidation of partially blocked derivatives by chromium(VI) oxide, by this method, DL-*xylo*-3-hexulose[10] was prepared. 3-Pentuloses can be prepared by transformation of aldopentoses in pyridine[3].

Secondary hydroxyl groups are specifically oxidized by mercury(II) acetate, even in the presence of unsubstituted primary alcoholic groups, and *erythro*-3-pentulose and DL-*erythro*-pentulose[11] were prepared by oxidation of ribitol Similarly, D-fructose and L-sorbose were prepared[12] from D-mannitol and D-glucitol, respectively.

This paper deals with the preparation of further pentuloses by the oxidation of D-arabinitol and xylitol by mercury(II) acetate, the oxidation of glycerol, ethylene glycol, and isopropyl alcohol is also described

DISCUSSION

Oxidation of D-arabinitol, xylitol, and ribitol[11] with mercury(II) acetate gave only pentuloses, thereby proving the selective oxidation of secondary hydroxyl groups On the other hand, glycolaldehyde is formed by oxidation of ethylene glycol, and

both dihydroxyacetone and DL-glyceraldehyde were obtained from glycerol These results provide evidence for the oxidation of secondary hydroxyl groups by mercury(II) acetate, and show that primary hydroxyl groups also undergo oxidation, although at a lower rate

The formation of the above products of oxidation passed through a maximum, probably because of subsequent dehydration of the resulting α-hydroxycarbonyl compounds[13] Since ketoses, which are formed to a greater extent, are dehydrated more readily than aldoses[14], it is evident that the effect of dehydration is pronounced and causes a big decrease in the yield of the α-hydroxycarbonyl compounds The structure of the dehydration products was not elucidated, but the formation of deoxypentosuloses may be presumed by analogy with the dehydration of hexuloses[13,15]

Since *erythro*-3-pentulose was not formed on oxidation of D-arabinitol, nor DL-*erythro*-pentulose and DL-*threo*-3-pentulose from xylitol, it is concluded that the initially formed pentuloses do not rearrange

Stoodley[11] assumed that the oxidation of ribitol with mercury(II) acetate proceeds through a 6-membered cyclic intermediate Such an intermediate is not possible for ethylene glycol and isopropyl alcohol, both of which are oxidised by the reagent However, the finding that the oxidations of particular, secondary hydroxyl groups do not proceed at the same rate, *e g*, in the case of D-arabinitol, indicates the operation of stereochemical effects It is worth noting that the yield of particular pentuloses need not necessarily reflect the extent of oxidation of individual secondary hydroxyl groups This can only be determined from a knowledge of the rate of dehydration of the various pentuloses Clearly, further investigation is necessary before an acceptable mechanism of oxidation of alditols can be presented

EXPERIMENTAL

Melting points were determined on a Kofler micro hotstage Solutions were evaporated under diminished pressure at 30–40°

Xylitol[16], D-*erythro*-pentulose[3], D-*threo*-pentulose[3], D-*threo*-3-pentulose[3], and *erythro*-3-pentulose[11] were prepared by literature methods All other chemicals were commercial products

Descending paper chromatography was performed on Whatman No 1 paper with (*a*) acetone–butyl alcohol–water (7 2 1) [the fraction of pentuloses ($R_P = 1$) was used as a reference for estimation of the particular spots, single pentuloses exhibited the same mobility in this solvent system]; (*b*) cyclohexanol–pyridine–water saturated with boric acid (6 5 2) and containing phenol (1 g/100 ml), the chromatography paper was soaked with 1% boric acid solution Detection was effected with aniline hydrogen phthalate[17] and potassium periodate–benzidine[18]

Preparative chromatography was performed on columns (90 × 4 5 cm) of Whatman cellulose or microcrystalline cellulose[19] Ion-exchange chromatography was effected on columns (200 × 1 cm) of Dowex-50W x8 (100–200 mesh) (Ba^{2+}) resin[20]

Polarographic determination of α-hydroxycarbonyl and α-dicarbonyl compounds was effected as previously described[21,22].

Oxidation of D-*arabinitol.* — A solution of D-arabinitol (10 g, 0.065 mole) and mercury(II) acetate (42 g, 0.13 mole) in absolute methanol (250 ml) was refluxed for 12 h. The insoluble mercury(I) acetate was filtered off, and washed with methanol. Mercury salts, precipitated from the filtrate by hydrogen sulphide, were filtered off and washed with methanol. The combined filtrates were evaporated, and the residual syrup was treated with absolute ethanol (75 ml). Unreacted D-arabinitol (4 g), which crystallized from this solution at 0°, was filtered off, and the filtrate was evaporated anew. According to paper chromatography [system (*a*)], the syrupy residue consisted of unreacted D-arabinitol (R_P 0.60), pentuloses, and three dehydration products (R_P 1.32, 1.52, and 1.76). This mixture was separated by cellulose column chromatography [solvent (*a*)] to give the dehydration products (1.05 g, 10.5%), pentuloses (1.71 g, 17.1%), and D-arabinitol. The mixture of pentuloses was fractionated by ion-exchange chromatography, with water as eluent, to give D-*threo*-pentulose (1.10 g, 11%), D-*threo*-3-pentulose (0.25 g, 2.5%), and D-*erythro*-pentulose (0.12 g, 1.2%).

When the reaction time of oxidation was extended to 24 h, 11% of oxidation products and 19.3% of dehydration products were isolated. When the molecular ratio of oxidation agent to D-arabinitol was changed to 4:1 for a reaction time of 12 h, 9.4% of oxidation products and 21.0% of dehydration products were obtained.

Oxidation of xylitol. — A solution of xylitol (10 g, 0.065 mole) and mercury(II) acetate (42 g, 0.13 mole) in absolute methanol (250 ml) was refluxed for 8 h and worked up as in the previous case, except that the unreacted xylitol did not crystallize. Paper chromatography [solvent (*a*)] showed that the reaction mixture contained unreacted xylitol (R_P 0.60), pentuloses, and two dehydration products (R_P 1.32 and 1.52).

The reaction mixture was first separated by means of ion-exchange chromatography, with water as eluent, to give DL-*threo*-pentulose and *erythro*-3-pentulose, both fractions were contaminated with dehydration products, as well as with unreacted xylitol. Each of these fractions was further separated by cellulose column chromatography [solvent (*a*)] to give dehydration products (1.78 g, 17.8%), DL-*threo*-pentulose (1.20 g, 12.0%), and *erythro*-3-pentulose (0.16 g, 1.6%).

Characterisation of pentuloses. — Pentuloses were identified by optical rotation and by the sign of their Cotton effect[23], by conversion into their crystalline hydrazones, and by paper chromatography [solvent (*b*)] of the pentitols resulting from reduction with sodium borohydride.

D-*threo*-Pentulose[5], syrup, $[\alpha]_D^{20}$ −32° (*c* 1.0, water), positive Cotton effect, 2,4-dinitrophenylhydrazone[3], m.p. 175–176° (chloroform) (Found: C, 39.87; H, 4.25; N, 17.05%. $C_{11}H_{14}N_4O_8$ (330.3) calc.: C, 40.00; H, 4.27; N, 16.96%). D-Arabinitol and xylitol were identified as reduction products.

D-*threo*-3-Pentulose, syrup, $[\alpha]_D^{20}$ −118° (*c* 1.0, water), was obtained in purer form than previously reported[23], negative Cotton effect, 2,5-dichlorophenyl-

hydrazone[3], m p 146–147° (chloroform) (Found. C, 42.46, H, 4 48; N, 9 00% $C_{11}H_{14}Cl_2N_2O_4$ (309.1) calc · C, 42 74; H, 4 57; N, 9 06%) D-Arabinitol was formed by the reduction

D-*erythro*-Pentulose, syrup, *o*-nitrophenylhydrazone[3], m.p 165–166° (ethanol); $[\alpha]_D^{20}$ −50° (*c* 0 2, methanol) (Found C, 46.52, H, 5.46, N, 14 95 $C_{11}H_{15}N_3O_6$ (285 3) calc . C, 46 32, H, 5 30, N, 14 73%) Ribitol and D-arabinitol were identified as reduction products

DL-*threo*-Pentulose, syrup, phenylosazone[24], m.p 205–207° (ethanol) (Found C, 62 37, H, 6 35; N, 17.05% $C_{17}H_{20}N_4O_3$ calc.: C, 62 15, H, 6.14, N, 17.06%) DL-Arabinitol and xylitol were identified as reduction products

erythro-3-Pentulose, syrup, 2,5-dichlorophenylhydrazone[11], m p 126–127° (chloroform) (Found· C, 42 49, H, 4.43, N, 9 01%. $C_{11}H_{14}Cl_2N_2O_4$ (309 1) calc C, 42 74, H, 4 57, N, 9.06%) Ribitol and xylitol were found to be the reduction products.

Oxidation of ethylene glycol — Ethylene glycol (2 g, 0 032 mole) was oxidized with mercury(II) acetate (20 g, 0 063 mole) in absolute methanol (100 ml) for 7 h Glycolaldehyde thus prepared was estimated polarographically[21], mercury salts being first precipitated by hydrogen sulphide, after 2 h, 2 4%; 4 h, 2 0%, 7 h, 1.4%.

Oxidation of glycerol — Glycerol (2 g, 0 022 mole) was oxidized with mercury-(II) acetate (14 g, 0 044 mole) in absolute methanol (100 ml) for 7 h The oxidation products (dihydroxyacetone and DL-glyceraldehyde), as well as pyruvaldehyde formed by dehydration, were estimated polarographically[22], the soluble mercury salts being first removed by hydrogen sulphide It was ascertained that the yield of dihydroxyacetone and DL-glyceraldehyde increased with time of oxidation up to *ca* 5 h (maximum, *ca.* 1.5% of each triose) The yield then decreased, with an accompanying increase of the dehydration product, *i e* , pyruvaldehyde

Oxidation of isopropyl alcohol. — Isopropyl alcohol (25 g, 0 42 mole) was subjected to oxidation with mercury(II) acetate (60 g, 0 19 mole) in absolute methanol (300 ml) for 32 h Acetone (3 5%) thus formed was determined gravimetrically as the 2,4-dinitrophenylhydrazone

Dehydration of pentuloses — Chromatographically pure pentuloses were refluxed in N methanolic acetic acid Two substances (R_P 1 32 and 1.52) were obtained by dehydration of D-*erythro*-pentulose. Two substances of similar R_P values were obtained from D-*threo*-pentulose, and one substance (R_P 1 76), in small proportion, from D-*threo*-3-pentulose. *erythro*-3-Pentulose underwent no detectable dehydration All of the above-mentioned substances were detected with potassium periodate–benzidine and by aniline hydrogen phthalate; an intense red coloration was formed, as with the dehydration products of pentitols formed by reaction with mercury(II) acetate.

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Note

The characterization of oligosaccharides attached to threonine and serine in a mannan glycopeptide obtained from the cell wall of yeast

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The structure of a glycopeptide isolated from the cell wall of yeast has been shown to be composed of (*1*) a highly branched mannan of high molecular weight, which is attached to the peptide moiety probably by a nitrogen glycosyl bond between 2-acetamido-2-deoxy-D-glucose and aspartamide, and (*2*) numerous monosaccharide or small oligosaccharide units joined to the peptide part of the molecule by glycosyl bonds on to the hydroxyl groups of serine and threonine[1] This communication reports the fractionation of the oligosaccharides which form the *O*-glycosyl-hydroxy-amino acid components of the glycopeptide, and the characterization of the disaccharides present The method used was a modification of a two-dimensional electrophoretic system employed in the identification of peptides[2] A separation of the components of the oligosaccharide mixture by molecular weight was achieved first by descending chromatography, and this was followed by an electrophoretic separation of the disaccharides in borate buffer

EXPERIMENTAL

Glycopeptide *A* was prepared from cell walls[1] of yeast and dissolved in 0 1N sodium hydroxide The solution was kept for 18 h at 21°, and this treatment with alkali resulted in a β-elimination reaction which released the oligosaccharides linked to the hydroxy-amino acids The alkali was then neutralized with acetic acid, and the solution was dialysed against distilled water for 18 h at 4° The diffusible material was desalted on a column of Zeo-karb SRC 225 (H^+) resin and freeze-dried Samples of the freeze-dried material, after hydrolysis by 2N hydrochloric acid, gave only a monosaccharide with the same mobility as mannose on a chromatogram developed with ethyl acetate–pyridine–water (8 2 1, by vol)

Oligosaccharide markers were prepared by acetolysis[3] of yeast mannan, the chloroform extraction and the charcoal decolorization were omitted, and sodium methoxide was used in place of barium methoxide

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Samples of the freeze-dried mixture of oligosaccharides released during the alkali treatment of glycopeptide *A*, and the oligosaccharides prepared from yeast mannan (marker) were chromatographed with butyl alcohol–ethanol–water (3:1.1, by vol), and detected with aniline hydrogen phthalate[4]. Four spots were detected from the oligosaccharides prepared from yeast mannan, these were identified as mono-, di-, and tri-saccharides Six spots, at least, were present in the material obtained from the glycopeptide, four of these had similar R_F values to those of the markers, the other two were near the origin and were higher oligosaccharides A transverse portion of the paper chromatogram containing the disaccharides obtained from both the glycopeptide and the yeast mannan was excised as a strip (6 5 cm wide) and sewn with a sewing machine, using a nylon thread, between two pieces of paper. so that the chromatographic strip was, at its centre, *ca* 12 cm from the cathode end of the electrophoretogram and 43 cm from the anode end (overall length, *ca* 56 cm) The paper was then wetted with 0 05M borate buffer (pH 9 2), in such a way that the buffer flowed evenly towards the strip from each side, and then subjected to electrophoresis in the same borate buffer at 40 volts/cm for 7 h The paper was dried at 60° and treated either with aniline hydrogen phthalate[4] acidified with acetic acid, or benzidine–trichloroacetic acid[5]

The marker showed the presence of two spots, in agreement with previous studies[6–8] The spot with the higher M_G value corresponded to 6-*O*-α-D-mannopyranosyl-D-mannose[6,7] The other probably was 2-*O*-α-D-mannopyranosyl-D-mannose[3 6 7] The weak colour which developed from this spot with aniline hydrogen phthalate agreed with the characteristic behaviour[9] of the (1→2) linkage.

The dimers obtained from the glycopeptide contained these two disaccharides, and a third one with a higher M_G value than that of 6-*O*-α-D-mannopyranosyl-D-mannose It was probably 3-*O*-α-D-mannopyranosyl-D-mannose, since this disaccharide has a large electrophoretic mobility[10] This dimer represents the only other linkage known to be present in yeast mannan[3]

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Note

The isolation of 6-kestose from the seeds of the horse chestnut (*Aesculus hippocastanum* L.)

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The sugar fraction of horse-chestnut seeds contains at least 13 compounds, including[1] D-glucose, sucrose, and maltose The remaining carbohydrates are of three types (1) amylosaccharide, (2) galactosylsucrose, and (3) fructosylsucrose. The amylose series contains the members D-glucose–maltooctaose, with the exception of maltohexaose The galactosylsucrose series is represented by stachyose and an unidentified pentasaccharide[2]

Of the three fructosylsucrose compounds, one was identified as 1-kestose[3], and the other was probably the tetrasaccharide We now report the identification of the third member as 6-kestose [**1**, *O*-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl α-D-glucopyranoside]

The fructosylsucroses have been found[4] among the products of the action of yeast invertase on sucrose, and the structure of 6-kestose has been determined[5] 6-Kestose, 1-kestose, and neokestose are found in many plants[6], and their role in plant metabolism is a subject of continuing interest

EXPERIMENTAL

All melting points are uncorrected Specific rotations were measured on a Zeiss polarimeter I r. spectra were obtained for Nujol mulls with a Zeiss UR-10 spectrometer. Colorimetric estimations were obtained with a Colorimeter M G F Berlin.

Isolation of sugar 3A. — The fraction rich in sugar 3A was obtained from the previously purified, aqueous, ethanolic extracts of horse-chestnut seeds[1]

A 10% aqueous solution containing 1 5 g of syrupy 3A was eluted from a mixture of active charcoal (Carbosorbid N, 15 g) and Celite 535 (15 g), using an over-pressure of 0 3 atm , successively with water, and 8%, 12%, and 15% ethanol The fractionation was monitored by t l c as shown in Table I.

Fractions VII and VIII were combined and evaporated under diminished pressure. Repeated crystallization of the residue from methanol gave 6-kestose as colourless, thin needles, m p 143–145° (dec), $[\alpha]_D^{20}$ +27° (*c* 1 2, water) [Found.

TABLE I

ISOLATION OF SUGAR 3A FROM A CHARCOAL–CELITE COLUMN

Fraction	*Eluent*	*Volume (ml)*	*Components*
I	water	100	—
II	8% ethanol	470	maltose
III	12% ethanol	25	trace of 1-kestose
IV	12% ethanol	100	1-kestose
V	12% ethanol	25	1-kestose
VI	12% ethanol	25	trace of 1-kestose, sugar 3A
VII	12% ethanol	75	sugar 3A
VIII	15% ethanol	50	sugar 3A
IX	15% ethanol	50	sugar 3A, traces of higher oligosaccharides
X	15% ethanol	100	higher oligosaccharides

C, 42 63; H, 6 34. Mol. wt (cryoscopy in water), 501 ±8. $C_{18}H_{32}O_{16}$ calc.: C, 42 87; H, 6 39% Mol. wt, 504] The X-ray data agreed with those reported by Binkley[7].

The R_F value of sugar 3A in t l c (propyl alcohol–cyclohexane–water–ethyl acetate, 9 2 2 3) was different from those of 1-kestose and neokestose

Periodate oxidation — Microscale periodate oxidation[8], in triplicate, of 6-kestose established a consumption of 3 87–3 96 mol of oxidant and the formation of 1 04–1 05 mol of formic acid. These data are consistent with structure **1**

Methylation — 6-Kestose (600 mg) was methylated according to the modified Haworth method[9] The final product (700 mg) was a pale-yellow syrup, having n_D^{20} 1 4603*, $[\alpha]_D^{20}$ +24° (*c* 0 6, water) (Found· C, 52 86; H, 8 60; OMe (micro-modified Klimowa method[10]), 51 76 $C_{29}H_{54}O_{16}$ calc C, 52 87, H, 8 26, OMe, 51 82%).

TABLE II

SEPARATION OF THE PRODUCTS OF HYDROLYSIS OF METHYLATED 6-KESTOSE ON COLUMNS OF SILICA GEL

Fraction	*Eluent*	*Volume (ml)*	*Qualitative contents*
I	Toluene–0 33% ethanol	200	—
II	Toluene–0 33% ethanol	550	Tetra-*O*-methylfructose
III	Toluene–0 33% ethanol	125	—
IV	Toluene–6% ethanol	250	—
V	Toluene–6% ethanol	250	Tetra-*O*-methylglucose
VI	Toluene–6% ethanol	400	Tetra-*O*-methylglucose (traces)
VII	Toluene–20% ethanol	300	Tri-*O*-methylfructose

The above product was hydrolysed as described previously[3], and the hydrolysate, which had $[\alpha]_D^{20}$ +19 3° (*c* 0 5, water), was added to a column (22 × 2 5 cm)

*The value cited by Albon *et al*[5] was erroneously printed, and the corrected value (1 4606) was kindly submitted by Dr Gross

of Polish silica gel[11] [25 g, deactivated by the addition of water (15 ml)] suspended in toluene (100 ml) containing 0 33% of ethanol as a 10% solution in the same solvent mixture Successive elution with toluene–0 33% ethanol, toluene–6% ethanol, and toluene–20% ethanol gave the results shown in Table II. The fractions were monitored by t l c [Kieselgel G (Merck), toluene–ethanol (4.1), detection with aniline hydrogen phthalate and orcinol reagents] Products corresponding to 2,3,4,6-tetra-*O*-methylglucose, 1,3,4,6-tetra-*O*-methylfructose, and 1,3,4-tri-*O*-methylfructose were the only compounds detected in fractions V, II, and VII, respectively

Evaporation of fraction II yielded 1,3,4,6-tetra-*O*-methylfructose (160 mg), n_D^{20} 1 4505, fraction V gave 2,3,4,6-tetra-*O*-methylglucose (170 mg), and fraction VII gave 135 mg of a colourless syrup, which crystallized from methanol to give 1,3,4-tri-*O*-methyl-D-fructose, m p 69–72°, lit [12], m p 68–71°.

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Note

Thermal degradation of 2-*O*-methylcellulose

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Several investigators[1] have suggested a 1,2-anhydro-4-*O*-substituted-α-D-glucopyranose intermediate in the thermal depolymerization of cellulose to 1,6-anhydro-β-D-glucopyranose; however, no direct and only limited indirect evidence has been presented to support the involvement of such an intermediate Our work on the polymerization of 1,6-anhydro-β-D-glucopyranose (levoglucosan) led us to examine the pyrolysis of 2-*O*-methylcellulose (**1**) This communication presents our findings and relates them to the thermal degradation of cellulose

Pyrolysis of **1** at 350° and 0 02 torr produced an amber, viscous distillate which, upon treatment with benzoyl chloride in pyridine, gave crystalline 1,6-anhydro-3,4-di-*O*-benzoyl-2-*O*-methyl-β-D-glucopyranose (**2a**) Deesterification of **2a** gave a crystalline, hygroscopic solid, 1,6-anhydro-2-*O*-methyl-β-D-glucopyranose (**2**), in an overall yield of 16% from cellulose Hydrolysis of **2** in 0 075M sulfuric acid gave 2-*O*-methyl-β-D-glucopyranose

The n m r spectrum of **2** (Fig 1) at 60 MHz in methyl sulfoxide-d_6 is similar to the spectrum of 1,6-anhydro-β-D-glucopyranose but differs from that of 1,6-

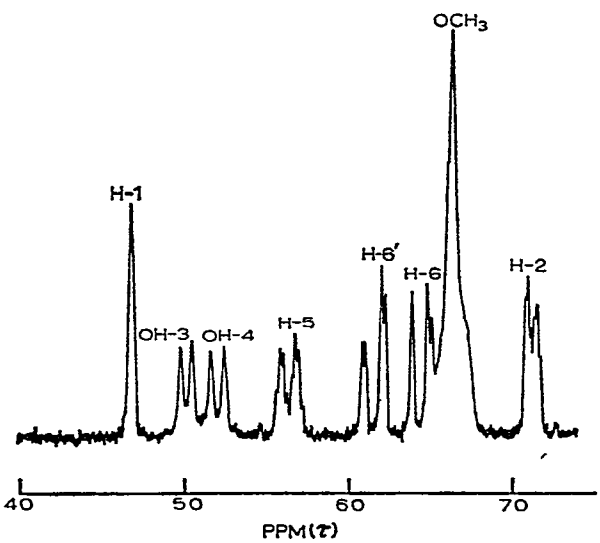


Fig 1 The 60-MHz n m r. spectrum of 1,6-anhydro-2-*O*-methyl-β-D-glucopyranose (**2**) in methyl sulfoxide-d_6

anhydro-β-D-glucofuranose. The sharp singlet at τ 6 62 was assigned to the methoxyl protons and the signal at lowest field (τ 4 67), which appeared as a slightly broadened singlet, was assigned to H-1. This shape for the signal of H-1 seems characteristic of several 1,6-anhydrohexopyranoses[2] and results from the minor splitting of the H-1 signal by vicinal coupling with H-2 and many additional, small-magnitude splittings caused by long-range coupling of H-1 with most, if not all[3], of the other ring protons

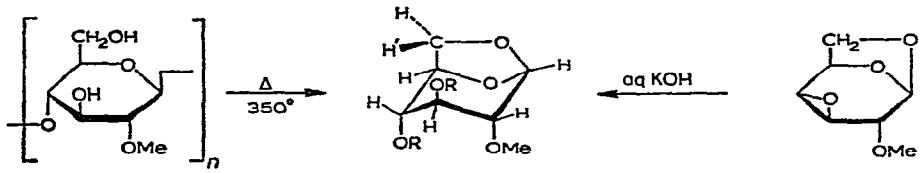
The assignments of the signals for H-5, H-6 (exo), and H-6′ (endo) in the spectrum of **2** are based on Hall's assignments[4] for these protons in 1,6-anhydro-β-D-glucopyranose The signal at τ 5 63, a doublet of narrow quartets, was assigned to H-5, the major splitting, which is due to coupling with H-6 ($J_{5\,6}$ = 6 0 Hz), is further separated into a total of eight lines indicating equal coupling of H-5 with H-6′, H-4, and H-3 ($J_{5,6'} \simeq J_{4,5} \simeq J_{3\,5} \simeq 1$ 2 Hz) The proton at C-5 might also be expected to show long-range coupling with H-1 through the pyranose ring-oxygen atom, but this coupling must be weak in **2** as it is[3] in 1,6-anhydro-2,3,4-tri-*O*-acetyl-β-D-mannopyranose ($J_{1,5} \simeq 0$ 1 Hz) The quartets at τ 6 15 and 6 48 ($J_{5\,6}$ = 1 2 Hz and $J_{6\,6}$ = 7.1 Hz) were assigned to H-6′ and H-6, respectively

The presence of the methoxyl group at C-2 causes the signal of H-2 to appear at higher field (τ 7 11), well apart from the unresolved signals of H-3 and H-4 (τ 6 52–6 73) In a similar manner, the H-1 signal of β-D-glucopyranose (τ 5 42)[5] appears at higher field when the hydroxyl group at C-1 is glycosidated to give methyl β-D-glucopyranoside (τ of H-1 is 5 65) The doublet of narrow triplets for the H-2 signal indicates coupling with H-3 ($J_{2\,3}$ = 3.3 Hz), which was removed upon irradiation at τ 6 58 (H-3), together with additional splitting of each principal line into a triplet by equal coupling with H-1 and H-4 ($J_{1,2} \simeq J_{2,4} \simeq 1$ 2 Hz) The magnitude[3] (~1 2 Hz) of the long-range couplings $J_{2,4}$ and $J_{3,5}$, together with the small value of $J_{2\,3}$ (3 3 Hz) are consistent with a *1C* (D) chair conformation of the pyranose ring in **2** As expected, substitution of a methoxyl group for a hydroxyl group in levoglucosan does not change the conformation of the pyranose ring of the molecule

The hydroxyl protons on C-3 and C-4 gave rise to a pair of doublets at τ 5 00 and 5 20, whereas the hydroxyl protons of levoglucosan gave signals at τ 5.11, 5 20, and 5 33 Irradiation at τ 6 58 (H-3) collapsed the doublet at τ 5 00 (OH-3) to a singlet and irradiation at τ 6 67 (H-4) changed the signal at τ 4 80 (OH-4) to a singlet The signals of the hydroxyl hydrogen atoms were eliminated from the spectrum by the addition[6] of a trace of hydrogen chloride to the methyl sulfoxide solution

Compound **2** was also prepared by another procedure (Scheme I) in an overall yield of 20% starting with 1,6-anhydro-β-D-glucopyranose By established procedures, levoglucosan was converted into 1,6 3,4-dianhydro-2-*O*-methyl-β-D-galactopyranose (**3**), which, upon refluxing in M aqueous potassium hydroxide, gave a compound in 55% yield that was identical in all respects to **2**

The thermal degradation of 2-*O*-methylcellulose to **2** indicates that a 1,2-anhydro-4-*O*-substituted-α-D-glucopyranose is probably not an intermediate in the pyrolysis of cellulose Assuming that pyrolysis proceeds by an ionic mechanism, a concerted process appears favorable in this reaction since charge dispersal in the



1 2 R=H 2a R=Bz 3

transition state cannot be stabilized by solvent molecules Anchimeric assistance by the oxygen atom at C-2, however, does not seem likely since three-membered oxonium-ion rings rarely occur[7]. Winstein, *et al* [8] have found that methoxyl groups do not anchimerically assist a leaving group if the transition state consists of a three-membered, cyclic, methyloxonium ion Olah and Bollinger[9], in their work on stable carbonium ions, have presented evidence that formation of a bridged, methyl-oxonium ion does not occur in the ionization of 2-halo-3-methoxy-2,3-dimethyl-butanes In addition, no evidence of C-2 participation *via* a bridged benzyloxonium ion was found by Ishikawa and Fletcher[10] in the methanolysis of 2-*O*-benzyl-3,4,6-tri-*O*-*p*-nitrobenzoyl-β-D-glucopyranosyl bromide On the other hand, anchimeric assistance by a methoxyl group is particularly favorable when the cyclic intermediate is five-membered[11] Numerous reports of five-membered, oxonium-ion intermediates have been made[12].

In the pyrolysis of cellulose, Kilzer and Broido[13] have postulated the formation of 1,4-anhydro-α-D-glucopyranose, a five-membered, cyclic intermediate, and its subsequent rearrangement to 1,6-anhydro-β-D-glucopyranose The thermal degradation[14] of a β-D-(1→4)-linked mannan gives 1,6-anhydro-β-D-mannopyranose, again probably through a 1,4-anhydro intermediate, since the reacting groups on carbon atoms C-1 and C-2 cannot assume a trans-diaxial arrangement Lemieux[15] has proposed the same intermediate in the alkaline transformation of phenyl β-D-mannopyranoside into 1,6-anhydro-β-D-mannopyranose Stable 1,4-anhydro derivatives of hexoses have been isolated[16], including 1,4-anhydro-2,3,6-tri-*O*-methyl-α-D-glucopyranose[17], prepared by the pyrolysis of 2,3,6-tri-*O*-methylcellulose

Based on considerable evidence favoring five-membered and not three-membered cyclic oxonium ions, it is more plausible that 2-*O*-methylcellulose, and probably cellulose, undergo thermal degradation through 1,4-anhydro intermediates

EXPERIMENTAL

General methods — Melting points were determined with a Thomas–Hoover melting point apparatus and are uncorrected N m r spectra were recorded in methyl sulfoxide-d_6 with a Varian A-60 spectrometer and the chemical shifts are given in τ values (p p m) from tetramethylsilane as the internal standard Spin-decoupling experiments were conducted by the "field-sweep" method[3]. Specific optical rotations were obtained with a Zeiss–Winkel polarimeter. All evaporations were done *in vacuo* below 50°

2-O-Methylcellulose (**1**) — 2-*O*-Methylcellulose (**1**) was prepared in 49% yield from acetate-grade, cotton linters by the procedure described by Falconer and Purves[18].

Anal Calc for cellulose having a degree of substitution of 0 13 nitrate and 0 95 methoxyl groups, and with sulfur present in elemental form OMe, 16 1, N, 0 99 Found. OMe, 16 04, 16 20, N, 0 96, 1 00, S, 1.89.

1,6-Anhydro-3,4-di-O-benzoyl-2-O-methyl-β-D-glucopyranose (**2a**) *Pyrolysis of 2-O-methylcellulose.* — Pyrolysis of **1** was performed on 0 5 g charges in a pyrex test tube (20 × 140 mm) having a 19/38 ground-glass joint A plug of glass wool was placed directly over the charge, and the test tube was fitted with a copper cooling-coil positioned externally about 50 mm from the charge The tube was positioned horizontally, and after evacuation to 0 02 torr, the end of the tube was inserted into a combustion furnace (Hoskens, Model FD 303A) kept at a constant temperature of 350° Immediately distillate began collecting on the cooled portion of the tube, and heating was continued for 1 h The distillate was dissolved in water, the solution was passed through Amberlite MB-3 (H^+, OH^-) resin, and the effluent from the column was evaporated, to give 0.27 g (54%) of an amber, viscous syrup The purified distillates from ten separate pyrolyses were combined to give 2 85 g of syrup, which was dissolved in 30 ml of anhydrous pyridine This solution was cooled to 4° and 9 ml of benzoyl chloride was added in 1-ml portions at 5-min intervals The reaction mixture was kept for 24 h at 4°, and allowed to stand an additional 5 h at room temperature Water (1 ml) was added to destroy excess benzoyl chloride, and, after 1 h at room temperature, 75 ml of 1,2-dichloroethane was added to the reaction mixture and the organic phase was washed successively with cold, 1 5M aqueous sulfuric acid, aqueous sodium hydrogen carbonate, and water The 1,2-dichloroethane solution was dried over sodium sulfate and evaporated to a syrup The product crystallized from a mixture of chloroform and methanol to give 3 89 g (62%) of material having m p 140–145° Recrystallization from methanol gave pure **2a**, m p 145 5–146 5°, $[\alpha]_D^{25}$ −192° (*c* 1 14, chloroform).

Anal Calc for $C_{21}H_{20}O_7$ (384 39) C, 65 62; H, 5 24 Found C, 65 79, H, 5 29

1,6-Anhydro-2-O-methyl-β-D-glucopyranose (**2**) — *A* 1,6-Anhydro-3,4-di-*O*-benzoyl-2-*O*-methyl-β-D-glucopyranose (3 82 g) was dissolved in chloroform (40 ml) to which was added anhydrous methanol (40 ml) To this solution was added 0 2 g of sodium dissolved in 10 ml of methanol and, after 1 h at room temperature, the reaction mixture was diluted with 75 ml of water The chloroform phase was extracted three times with distilled water, the combined aqueous phases were passed through an Amberlite MB-3 (H^+, OH^-) resin, and the effluent was concentrated to a syrup (2 06 g) A small portion of the syrup was distilled under diminished pressure at 1 torr in a microsublimation apparatus to give a distillate that crystallized A solution of the syrup in a mixture of acetone and hexane was seeded and kept at −10° The first crop of crystals, 1 65 g (95%), m p 92–94°, was recrystallized twice from acetone–hexane to give pure **2**, m p 93–94°, $[\alpha]_D^{25}$ −72 7° (*c* 1 45, acetone)

Anal Calc for $C_7H_{12}O_5$ (176 17) C, 47 73, H, 6 87 Found C, 47 93; H, 6 69.

B. 1,6 3,4-Dianhydro-2-*O*-methyl-β-D-galactopyranose (**3**), 0 309 g, m p 91–92°, prepared as described by Černy, *et al* [19], was refluxed for 14 h in M aqueous potassium hydroxide solution (25 ml), at which time t l c (developer ethyl acetate, and visualization by charring with sulfuric acid) showed complete consumption of **3**. The reaction mixture was diluted with water (20 ml) neutralized by passage through Amberlite MB-3 (H^+, OH^-) resin, and concentrated to give a colorless syrup. Compound **2** was crystallized as before from acetone–hexane to give 0 243 g (70 5%) of material having m p 84–87° Two recrystallizations from ethyl acetate gave a material whose n m r spectrum, specific optical rotation, m p and mixed m p were identical with those of **2** prepared in part *A*

Hydrolysis of 1,6-anhydro-2-O-methyl-β-D-glucopyranose (**2**) — 1,6-Anhydro-2-*O*-methyl-β-D-glucopyranose (0 19 g) was refluxed for 72 h with 30 ml of 0 075M sulfuric acid The solution was cooled, neutralized by passage through Amberlite MB-3 (H^+, OH^-) resin, and concentrated to a syrup, yield 0 11 g (52%) The product was crystallized from ethyl acetate at −10° (after seeding) to give 0 056 g (27%) of material having m p 155 5–156 5°. A mixed m p with authentic 2-*O*-methyl-β-D-glucopyranose (155–157°) was essentially undepressed (m p 153–156°) and the n m r spectra of both samples in deuterium oxide were identical, n m r data with reference to sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard τ 4 58 (doublet, H-1_α), 5 37 (doublet, H-1_β), 6 15–6 33 (multiplet), 6 41, 6 53 (singlets, methoxyl), and 6 43–6 75 (multiplet)

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Note

Glycosyl chlorides through reaction with zinc chloride–thionyl chloride*

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The synthesis of 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl chloride[1] (**2**) is often accompanied by decomposition Degradation is partially attributed to initial cleavage of benzyl ether groups Thus, in the hands of the authors, several chromatographically distinct zones were found to result from the treatment of 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranose[2] (**1**) with thionyl chloride at 70°[1]. These by-products are undesirable in the subsequent Koenigs–Knorr condensation of the syrupy glycosyl chloride because they tend to make isolation of the product difficult. It was found that zinc chloride in the presence of thionyl chloride considerably speeds up the chlorination reaction with a minimum of side products Complete conversion of **1** into **2** in 0 5 h at room temperature occurs without degradation Thionyl chloride alone at room temperature does not effect complete reaction with **1** even within two weeks Less than a 1 1 ratio of zinc chloride–thionyl chloride also effects the reaction, but optimum yield is obtained with roughly equimolar quantities A slight excess of zinc chloride was used to ensure complete formation of a complex in the presence of traces of atmospheric moisture

Although halogenation of alcohols with zinc chloride–hydrogen chloride has been utilized for some time[3,4], halogenation of alcohols with thionyl chloride is more usually carried out in the presence of heterocyclic bases such as pyridine or 2,6-lutidine[3,5]. Use of a zinc chloride–thionyl chloride complex in this chlorination reaction was suggested by one of the authors (T. G Squires, unpublished data) through previous experience in chlorinating a variety of alcohols In addition, the esterification of alcohols with sulfonyl chlorides is enhanced by zinc chloride The mechanism and stereospecificity of the reaction are being studied in light of other Lewis acid–base interactions with oxychlorides[6,7] A recent publication[8] utilized a 1 1 zinc chloride–phosphoryl chloride complex to form a powerful Lewis acid in a reaction somewhat analogous to that reported here

Assignment of the α-D configuration to **2** was made by comparison with the glycosyl chloride of Austin and coworkers[1], through optical rotatory power and n.m.r. spectral data. The small coupling of H-1 and H-2 ($J_{1,2}$ = 3 Hz) indicates a

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cis relationship for these protons resulting from an α-D anomeric configuration[9] The chlorination reaction occurs with overall retention of anomeric configuration

Crystalline methyl 2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranoside was isolated in good yield from the reaction of **2** with sodium methoxide in methanol The physical constants for this glycoside are identical with those reported by Austin and coworkers[1]

EXPERIMENTAL

Ascending t l c was performed on Silica Gel G (E Merck Co., Darmstadt, Germany, activated for 1 h at 110°) and Eastman Chromagram sheet 6060 with fluorescent indicator Location of zones was achieved with iodine, u v light, or by charring after spraying with sulfuric acid Solvents for t l c were 1 1 benzene–ethyl ether (solvent *A*) and 19 1 petroleum ether (30–60°)–methanol (solvent *B*) Melting points were determined in capillaries with a Mel-Temp apparatus and are uncorrected I r. spectra were recorded on a Beckman Model IR5 spectrometer with sodium chloride windows or potassium bromide pellets N m r spectra were recorded at 60 MHz on a Varian A-60 spectrometer, with saturated solutions in chloroform-*d* Microanalyses were performed by Galbraith Laboratories, Knoxville, Tennessee 37921.

2,3,4,6-*Tetra*-O-*benzyl*-α-D-*glucopyranosyl chloride* (**2**) — Preparation of **1** was carried out by Schmidt's procedure[2] A quantity of **1** (0 8 g, 1 4 mmoles) was dissolved in benzene (50 ml) contained in a round-bottomed flask equipped with a calcium chloride drying-tube and magnetic stirrer Zinc chloride (2 14 g, 15 mmoles) was added and thionyl chloride (1 91 g, 11 mmoles) was mixed in, with care being taken to exclude atmospheric moisture The reaction was monitored by t l c During the reaction only two zones were present, R_F 0 87 (**2**) and R_F 0 67 (**1**) (solvent *A*, u v and sulfuric acid detection) When t l c indicated disappearance of **1** (0 5 h), the reaction mixture was passed through a 3-cm thickness of silica gel on a sintered-glass funnel under suction The filter was washed several times with benzene until no more Molisch-positive[10] material dripped from the funnel The filtrate was concentrated on a rotary evaporator and the residue was coevaporated three times with benzene (50 ml) at 40° to afford **2** as a colorless syrup, which was pumped for 2 h at 40° under vacuum (0 5 mm) to remove the last traces of solvent, yield 0 65 g (74%), $[\alpha]_D^{23}$ +109° (*c* 1, benzene), +62° (*c* 1, chloroform), n m r. data for **1**. τ 2 72 (20 protons, phenyl-ring protons), τ 4 75 (1 proton doublet, $J_{1\,2}$ 3 Hz, H-1), τ 6 48 (1-proton triplet, assigned to H-2 by decoupling from H-1), τ 5 10–6 61 (14 protons, multiplet, carbohydrate ring protons and benzylic-CH_2-), n m r data for **2** τ 3 90 (1-proton doublet, $J_{1\,2}$ 3 Hz, H-1), other chemical shifts for **2** were essentially as for **1** except for the absence of an anomeric-proton signal at τ 4 75; no hydroxyl absorption in the i r spectrum of **2**

Anal Calc for $C_{34}H_{35}ClO_5$ C, 73 21, H, 6 32; Cl, 6 34. Found C, 73 25; H, 6 31, Cl, 6 55

The product **2** was chromatographically homogeneous in solvents *A* (R_F 0 87)

and *B* (R_F 0.74). Reaction of **2** with sodium methoxide–methanol was effected according to the method of Austin and coworkers[1]. Quantitative conversion of **2** was noted by t.l.c., and the product, yield 43%; m.p. 69–70° (EtOH), $[\alpha]_D^{22}$ +10.5° (*c* 1.7, $CHCl_3$), R_F 0.63 (solvent *B*) had physical data in accord with those previously reported[1] for methyl 2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranoside.

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Note

Solvent effects on formation of the starch–iodine complex

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INTRODUCTION

Ono *et al* [1] found that in methyl sulfoxide–water solutions containing less than 28 moles of water per liter a positive starch–iodine test could not be obtained In order to study the factors involved in determining whether a positive test is obtained in the presence of nonaqueous solvents we have investigated the solvent requirements necessary to obtain a positive test in aqueous solutions of the following solvents. methyl sulfoxide, *N*-methylacetamide, *N*,*N*-dimethylformamide, acetone, *p*-dioxane, methanol, and ethanol

DISCUSSION

No general minimum requirement of water necessary for a positive test was found The percentage of water necessary for a positive test varies from 39% with *N*-methylacetamide to 68% (v/v) with acetone (Table I) These data indicate that the critical factor is not some minimum proportion of water The water requirement is not directly related to either the dipole moment or the dielectric constant of the nonaqueous solvent used The i r spectrum of an aqueous solution of the starch–iodine complex was similar to that of the starch solution alone

TABLE I

Solvent	*Minimum % of water (v/v) to give color*	*Dipole moment, μ*
N-Methylacetamide	39	3 7
Methanol	47	1.7
Ethanol	52	1 7
Methyl sulfoxide	53	3 9
N,*N*-Dimethylformamide	58	3 8
p-Dioxane	58	0
Acetone	68	2 9

The effects of the different solvents on the amounts of water required for color formation can be interpreted in terms of the loose "deformed helix" of Ono and

its conversion into a rigid helix when formation of a complex takes place. It can be assumed that the loose helix in aqueous solution is hydrated and that the same is true of the rigid helix of the complex This picture is further supported by the viscosity studies of Banks and Greenwood[2], which indicate that the amylose molecule behaves as a random coil in methyl sulfoxide solution

In solutions containing solvents other than water the other solvent competes with water for the solvation sites on the loose coil. In the tight or rigid coil there is room between one turn of the coil and the next turn for only a small molecule (such as water) to solvate, and hence some water is required On the other hand, in the loose coil there is adequate room between turns of the coil for larger molecules to solvate the amylose, and if these larger molecules have sufficient solvating power (as might be indicated by their having a high dipole moment), the proper steric fit of the larger solvating molecule between turns of the coil should actually tend to stabilize the amylose molecule in the loose coil To the extent that the loose coil is stabilized by the solvating molecule it will be that much more difficult for the deformed helix to change over to the rigid helix and therefore, that much more difficult to form the colored complex. Our data, although limited, would indicate that there may be some optimum size for the solvent molecule which gives the best stabilization of the deformed helix

Size can not be considered alone since obviously the dipole moment has an important effect on solvating power It is possible to interpret the results in terms of the size and dipolar character of the various solvent molecules Thus the molecule of acetone ($\mu = 2.9$) would appear to be of a near optimum size for fitting into the deformed helix The combination of suitable size with a high dipole moment makes acetone a solvent that requires a minimum of 68% of water to permit formation of the rigid helix and the colored complex The other solvents having high dipole moments, methyl sulfoxide and *N,N*-dimethylformamide, require 53 and 58% of water, respectively, for the complex to form These molecules are a little larger than acetone and may not fit into the deformed helix in a manner that would stabilize the deformed helix as well as acetone would Methanol and ethanol, while smaller, do not have such large dipole moments and hence do not solvate the starch as well as most of the other solvents studied

Two of the solvents, *p*-dioxane and *N*-methylacetamide, require special comment *p*-Dioxane has a zero dipole moment because this observed moment is the resultant of two opposing dipoles. If we consider that only one of these dipoles would be involved in solvating the starch molecule, then it gives a better picture of the true situation to consider only one-half of the *p*-dioxane molecule and to approximate its solvating power as being somewhat like methyl ether ($\mu = 1.3$). On this basis, *p*-dioxane appears to fit well into the deformed helix and its dipole (for one end of the molecule) is sufficient to give good stabilization of the deformed helix.

The low percentage of water required to allow formation of a complex in *N*-methylacetamide indicates either a low degree of stabilization of the deformed helix or, alternatively, that the rigid helix necessary for formation of the complex

can be partly solvated by *N*-methylacetamide Evidence favors the latter view. This solvent is known to form strong hydrogen bonds and may be doing so either with the rigid coil directly or as the second shell of solvating molecules around the coil, with water molecules forming the first shell. A comparison of the visible spectra of the starch–iodine complexes in the various solvents supports the idea that the molecules of *N*-methylacetamide are involved directly in the starch–iodine complex in that solvent. The complex formed in the other solvents studied shows a single maximum at 546–596 nm (depending on the solvent) while the complex in *N*-methylacetamide shows three maxima at 449, 546, and 675 nm

EXPERIMENTAL

Solutions of starch (1% w/v, 2 4 ml) with various amounts of added organic solvent were tested for color formation by the addition of 1 drop of iodine solution (prepared from 60 g of potassium iodide and 40 g of iodine in 1 liter of solution) Any formation of a blue color was taken as a positive test.

Visible spectra were taken in 1-cm cells on a Perkin–Elmer Model 202 spectrophotometer. The i r spectrum was taken in a 0.05 mm AgCl cell with a Beckmann Model IR-8 spectrophotometer

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